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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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AUGUST, 1977

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THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

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Vol. 153, No. 1 August, 1977

THE
BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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II. CERTIFICATE OF ORGANIZATION

(On File in the Office of the Secretary of the Commonwealth)

No. 3170

We, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, and William T. Sedgwick, Edward G. Gardiner, Susan Mims and Charles Sedgwick Minot being a majority of the Trustees of the Marine Biological Laboratory in compliance with the requirements of the fourth section of chapter one hundred and fifteen of the Public Statutes do hereby certify that the following is a true copy of the agreement of association to constitute said Corporation, with the names of the subscribers thereto:-

We, whose names are hereto subscribed, do, by this agreement, associate ourselves with the intention to constitute a Corporation according to the provisions of the one hundred and fifteenth chapter of the Public Statutes of the Commonwealth of Massachusetts, and the Acts in amendment thereof and in addition thereto.

The name by which the Corporation shall be known is THE MARINE BIOLOGICAL LABORATORY.

The purpose for which the Corporation is constituted is to establish and maintain a laboratory or station for scientific study and investigations, and a school for instruction in biology and natural history.

The place within which the Corporation is established or located is the city of Boston within said Commonwealth.

The amount of its capital stock is none.

In Witness Whereof, we have hereunto set our hands, this twenty seventh day of February in the year eighteen hundred and eighty-eight, Alpheus Hyatt, Samuel Mills, William T. Sedgwick, Edward G. Gardiner, Charles Sedgwick Minot, William G. Farlow, William Stanford Stevens, Anna D. Phillips, Susan Mims, B. H. Van Vleck.

That the first meeting of the subscribers to said agreement was held on the thirteenth day of March in the year eighteen hundred and eighty-eight.

In Witness Whereof, we have hereunto signed our names, this thirteenth day of March in the year eighteen hundred and eighty-eight, Alpheus Hyatt, President, William Stanford Stevens, Treasurer, Edward G. Gardiner, William T. Sedgwick, Susan Mims, Charles Sedgwick Minot.

(Approved on March 20, 1888 as follows:

I hereby certify that it appears upon an examination of the within written certificate and the records of the corporation duly submitted to my inspection, that the requirements of sections one, two and three of chapter one hundred and fifteen, and sections eighteen, twenty and twenty-one of chapter one hundred and six, of the Public Statutes, have been complied with and I hereby approve said certificate this twentieth day of March A.D. eighteen hundred and eighty-eight.

CHARLES ENDICOTT
Commissioner of Corporations)

III. ARTICLES OF AMENDMENT

(On file in the office of the Secretary of the Commonwealth)

We, James D. Ebert, President, and David Shepro, Clerk of the Marine Biological Laboratory, located at Woods Hole, Massachusetts 02543, do hereby certify that the following amendment to the Articles of Organization of the Corporation was duly adopted at a meeting held on August 15, 1975, as adjourned to August 29, 1975, by vote of 444 members, being at least two-thirds of its members legally qualified to vote in meetings of the corporation:

VOTED: That the Certificate of Organization of this corporation be and it hereby is amended by the addition of the following provisions:

> "No Officer, Trustee or Corporate Member of the corporation shall be personally liable for the payment or satisfaction of any obligation or liabilities incurred as a result of, or otherwise in connection with, any commitments, agreements, activities or affairs of the corporation.
>
> "Except as otherwise specifically provided by the Bylaws of the corporation, meetings of the Corporate Members of the corporation may be held anywhere in the United States.
>
> "The Trustees of the corporation may make, amend or repeal the Bylaws of the corporation in whole or in part, except with respect to any provisions thereof which shall by law, this Certificate or the Bylaws of the corporation, require action by the Corporate Members."

The foregoing amendment will become effective when these articles of amendment are filed in accordance with Chapter 180, Section 7 of the General Laws unless these articles specify, in accordance with the vote adopting the amendment, a later effective data not more than thirty days after such filing, in which event the amendment will become effective on such later date.

In Witness whereof and Under the Penalties of Perjury, we have hereto signed our names this 2nd day of September, in the year 1975, James D. Ebert, President; David Shepro, Clerk.

(Approved on October 24, 1975, as follows:

I hereby approve the within articles of amendment and, the filing fee in the amount of $10 having been paid, said articles are deemed to have been filed with me this 24th day of October, 1975.

PAUL GUZZI
Secretary of the Commonwealth)

IV. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised February 13, 1976)

I. (A) The name of the Corporation shall be The Marine Biological Laboratory. The Corporation's purpose shall be to establish and maintain a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history.

(B) Marine Biological Laboratory admits students without regard to race, color, sex, national and ethnic origin to all the rights, privileges, programs and activities generally accorded or made available to students in its courses. It does not discriminate on the basis of race, color, sex, national and ethnic origin in employment, administration of its educational policies, admissions policies, scholarship and other programs.

II. (A). The members of the Corporation ("Members") shall consist of persons elected by the Board of Trustees, upon such terms and conditions and in accordance with such procedures, not inconsistent with law or these Bylaws, as may be determined by said Board of Trustees. Except as provided below, any Member may vote at any meeting, either in person or by proxy executed no more than six months prior to the date of such meeting. Members shall serve until their death or resignation unless earlier removed, with or without cause, by the affirmative vote of two-thirds of the Trustees then in office. Any Member who has attained the age of seventy years or has retired from his home institution shall automatically be designated a Life Member provided he signifies his wish to retain his membership. Life Members shall not have the right to vote and shall not be assessed for dues.

(B) The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

III. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk, elected or appointed by the Trustees as set forth in Article IX.

IV. The Annual Meeting of the Members shall be held on the Friday following the Second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 a.m. Subject to the provisions of Article VIII(2), at such meeting the Members shall choose by ballot six Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the Members may be called by the Chairman or Trustees to be held at such time and place as may be designated.

V. Twenty-five Members shall constitute a quorum at any meeting. Except as otherwise required by law or these Bylaws, the affirmative vote of a majority of the Members voting in person or by proxy at a meeting attended by a quorum (present in person or by proxy) shall constitute action on behalf of the Members.

VI. (A) Inasmuch as the time and place of the Annual Meeting of Members are fixed by these Bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of Members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least 15 days before such meeting, to each Member at his or her address as shown on the records of the Corporation.

(B) Any meeting of the Members may be adjourned to any other time and place by the vote of a majority of those Members present or represented at the meeting, whether or not such Members constitute a quorum. It shall not be necessary to notify any Member of any adjournment.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated. Notice of Trustees' meetings may be given orally, by telephone, telegraph or in writing; and notice given in time to enable the Trustees to attend, or in any case notice sent by mail or telegraph to a Trustee's usual or last known place of residence, at least one week before the meeting shall be sufficient. Notice of a meeting need not be given to any Trustee if a written waiver of notice, executed by him before or after the meeting is filed with the records of the meeting, or if he shall attend the meeting without protesting prior thereto or at its commencement the lack of notice to him.

VIII. (A) There shall be four groups of Trustees:

(1) Trustees (the "Corporate Trustees") elected by the Members according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Trustees shall be divided into four classes of six, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms.

(2) Trustees ("Board Trustees") elected by the Trustees then in office according to such procedures, not inconsistent with these Bylaws, as the Trustees shall have determined. Except as provided below, such Board Trustees shall be divided into four classes of three, one class to be elected each year to serve for a term of four years. Such classes shall be designated by the year of expiration of their respective terms. It is contemplated that, unless otherwise determined by the Trustees for good reason, Board Trustees shall be individuals who have not been considered for election as Corporate Trustees.

(3) Trustees ex officio, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(4) Trustees emeriti who shall include any Member who has attained the age of seventy years (or the age of sixty-five and has retired from his home institution) and who has served a full elected term as a regular Trustee, provided he signifies his wish to serve the Laboratory in that capacity. Any Trustee who qualifies for emeritus status shall continue to serve as a regular Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Members or by the Board, as the case may be. The Trustees ex officio and emeriti shall have all the rights of the Trustees, except that Trustees emeriti shall not have the right to vote.

(B) The aggregate number of Corporate Trustees and Board Trustees elected in any year (excluding Trustees elected to fill vacancies which do not result from expiration of a term) shall not exceed nine. The number of Board Trustees so elected shall not exceed three and unless otherwise determined by vote of the Trustees, the number of Corporate Trustees so elected shall not exceed six.

(C) The Trustees and Officers shall hold their respective offices until their successors are chosen in their stead.

(D) Any Trustee may be removed from office at any time with or without cause, by vote of a majority of the Members entitled to vote in the election of Trustees;

or for cause, by vote of two-thirds of the Trustees then in office. A Trustee may be removed for cause only if notice of such action shall have been given to all of the Trustees or Members entitled to vote, as the case may be, prior to the meeting at which such action is to be taken and if the Trustee so to be removed shall have been given reasonable notice and opportunity to be heard before the body proposing to remove him.

(E) Any vacancy in the number of Corporate Trustees, however arising, may be filled by the Trustees then in office unless and until filled by the Members at the next Annual Meeting. Any vacancy in the number of Board Trustees may be filled by the Trustees.

(F) A Corporate Trustee or a Board Trustee who has served an initial term of at least 2 years duration shall be eligible for re-election to a second term, but shall be ineligible for re-election to any subsequent term until two years have elapsed after he last served as a Trustee.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall elect a Treasurer and Clerk to serve one year, and Board Trustees as described in Article VIII (B). They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents of the Corporation and may remove them at any time. They may fill vacancies occurring in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number as provided in Article X, and to delegate to such Committee such of their own powers as they may deem expedient in addition to those powers conferred by Article X. They shall from time to time elect Members to the Corporation upon such terms and conditions as they shall have determined, not inconsistent with law or these bylaws.

X. (A) The Executive Committee is hereby designated to consist of not more than ten members, including the ex officio Members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and the affirmative vote of a majority of those voting at any meeting at which a quorum is present shall constitute action on behalf of the Executive Committee. The Executive Committee shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by vote of the Board or by law.

(D) The Executive Committee shall keep appropriate minutes of its meetings and its action shall be reported to the Board of Trustees.

(E) The elected Members of the Executive Committee shall constitute as a standing "Committee for the Nomination of Officers", responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

XI. A majority of the Trustees then in office shall constitute a quorum. A lesser number than a quorum may adjourn any meeting from time to time without further notice.

XII. Any action required or permitted to be taken at any meeting of the Trustees or of the Executive Committee may be taken without a meeting if all the Trustees or members of the Executive Committee, as the case may be, consent to the action in writing and such written consents are filed with the records of meetings. Such a consent shall be treated for all purposes as a vote at a meeting.

XIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees then in office.

XIV. These Bylaws may be amended by the affirmative vote of the Members at any meeting, provided that notice of the substance of the proposed amendment is stated in the notice of such meeting. As authorized by the Articles of Organization, the Trustees, by a majority of their number then in office, may also make, amend, or repeal these Bylaws, in whole or in part, except with respect to (a) the provisions of these Bylaws governing (i) the removal of Trustees and (ii) the amendment of these Bylaws and (b) any provisions of these Bylaws which by law, the Articles of Organization or these Bylaws, requires action by the Members.

No later than the time of giving notice of the meeting of Members next following the making amending or repealing by the Trustees of any Bylaw, notice thereof stating the substance of such change shall be given to all Corporation Members entitled to vote on amending the Bylaws.

Any Bylaw adopted by the Trustees may be amended or repealed by the Members entitled to vote on amending the Bylaws.

XV. The account of the Treasurer shall be audited annually by a certified public accountant.

XVI. The Corporation will indemnify every person who is or was a trustee, officer or employee of the Corporation or a person who provides services without compensation to an Employee Benefit Plan maintained by the Corporation, for any liability (including reasonable costs of defense and settlement) arising by reason of any act or omission affecting an Employee Benefit Plan maintained by the Corporation or affecting the participants or beneficiaries of such Plan, including without limitation any damages, civil penalty or excise tax imposed pursuant to the Employee Retirement Income Security Act of 1974; provided, (1) that the Act or omission shall have occurred in the course of the person's service as trustee or officer of the Corporation or within the scope of the employment of an employee of the Corporation or in connection with a service provided without compensation to an Employee Benefit Plan maintained by the Corporation, (2) that the Act or omission be in good faith as determined by the Corporation (whose determination made in good faith and not arbitrarily or capriciously shall be conclusive), and (3) that the Corporation's obligation hereunder shall be offset to the extent of any otherwise applicable insurance coverage, under a policy maintained by the Corporation or any other person, or other source of indemnification. (August 13, 1976).

V. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

As the Laboratory enters its ninetieth year, its objectives have not changed one whit from those set forth by its founders. It remains a center whose primary function is to provide opportunities and to serve as an unexcelled setting for innovation. It recognizes, as Caryl Haskins put it, "the fundamental individuality of innovation and the narrow human and material bases whence it springs." It stresses the uniqueness of discovery at a time when information and originality are often confused.

It has always been so at MBL, but until a scant decade ago the Laboratory's resources were limited largely to summer investigators and students. The need to develop year-round research and teaching programs, while holding fast to the best features of the renowned summer offerings, was enunciated clearly in 1966 in Philip B. Armstrong's final report, when he wrote "... the Trustees of the Laboratory have been keenly interested in realizing the full potentialities of the Laboratory not only during the summer months but during the rest of the year." In the following year, his successor, H. B. Steinbach, observed that "The use of our facilities throughout the year continues to grow," and he went on to announce the formation of a committee to "explore the possibility of instituting special training programs and of finding an individual who might give general direction to implementing the plans."

While it is clear that the Laboratory's policy has been established by a series of Executive Committees and four Directors over a decade, the potentialities immanent in its resources and rich tradition have been realized only slowly—much too slowly. The reasons are manifold, not the least being the dwindling of the Nation's material resources for science. Others are less obvious, but possibly more crucial: the confusion between the need to protect the original mind in whatever guise it appears and "personal privilege," and between intellectual elitism and "clubbishness," resulting in corporate inertia and in a preoccupation with the smaller, rather than the larger problems. Thus we inch forward, rather than move ahead with determination.

The challenges to the Laboratory remain grave. The stakes are high, involving the continued development of our total resources. Although in the period under review, July 1, 1976 through mid-May, 1977 we have made significant progress on several fronts, including an improvement in our financial outlook as revealed in our Treasurer's Report, we could be in serious danger of regression if we do not move forward with verve.

I would emphasize that such progress as can be reported in the sections to follow has accrued during the tenure of two Directors, Keith Porter (until December 31, 1976) and myself.

A center for postgraduate and continuing education

In recent years the Laboratory has been one of the first centers to recognize and address a problem of increasing national concern, the failure of young investigators to play significant roles in research beyond their immediate postdoctoral years. The transition from postdoctoral fellow or trainee to independent scientist is a "sensitive period" during which young scientists' careers can be shaped and often lifelong research careers established. Our *Steps Toward Independence Program* has evolved to meet this need. Untenured young faculty members from the Nation's universities are furnished the facilities, associations and training needed for them to apply and develop their research abilities, enabling them to assume responsibilities as independent scientists.

In recent summers, thanks to the generous support of a number of foundations, the Laboratory has been able to provide opportunities for eight to ten young investigators—eight in 1976, with another eight anticipated in 1977. Now thanks to an award from the National Science Foundation's RIAS (Research Initiation and Support) Program, these numbers will be increased to twelve in 1978, increasing to twenty-four by 1980.

The Steps Toward Independence Program has been coordinated with the recently established *Macy Scholars Program* which provides similar opportunities for younger faculty members and students in predominantly black institutions.

Since 1966 the Josiah Macy, Jr. Foundation has been deeply committed to efforts to increase minority representation in medicine and the related health professions. The Foundation's programs have played a significant role in bringing more members of the minority groups, particularly blacks, into the medical profession. As a result of its experience over the past decade, the Foundation concluded that its first priority in this area should be to replenish and expand the minority group pool of potential applicants. Therefore, it has begun to concentrate on programs designed to strengthen preparation for medicine and the related health professions, especially within the historically black colleges where the greatest reservoir of talent is developed.

Frequently, biology programs being offered in such colleges do not include quantitative courses at cellular and molecular levels. One way of strengthening the curricula of such institutions is to provide selected faculty members with intensive courses in quantitative biology and with opportunities to renew their interests in research in an environment providing opportunity for enrichment in the basic sciences underpinning modern medicine.

Since 1971, the Foundation has been sponsoring summer institutes for health professions advisors and biology teachers. In 1976 the Foundation announced a grant to MBL to support a program to support blacks and members of other minority groups in modern biology and biomedicine. Entrance to the program may be through any of several routes: one of the summer courses at MBL, a January short-term course, the MBL's Year in Science program, or a semester or a year in the Boston University Marine Program. Depending on the previous experience of the participant, after two summer courses or a short-term course and a summer course, an individual's program would be capped by at least a summer in the Laboratory's Steps Toward Independence Program.

The summer of 1976 found eleven Macy Scholars in residence, five as independent investigators and six as students in courses. Another nine graduate and undergraduate students were enrolled in January 1977, short-term courses, and as many as fifteen Macy Scholars are anticipated in the summer of 1977—six investigators, one junior investigator, and possibly eight students in courses.

These programs are now an integral part of the life of the Laboratory. They are not, however, what Steinbach and his committee envisioned a decade ago when the Director spoke of "special training programs." They had in mind intensive one- and two-week programs throughout the year intended to provide ten to twenty professional scientists with a working knowledge of techniques on timely new subjects. Ten years after that committee (which was chaired by the late Mac V. Edds, Jr.) made its principal recommendations, the program is at last underway, and an invitation extended to a *Coordinator of Continuing Education*, Dr. Morton Maser, who is expected to take up residence on July 1, 1977. His responsibilities will include the development of six to ten short courses annually (the ambitious course offered in the spring of 1977, "Electron Microscopy in the Biological Sciences" is a prototype) and the management of the Steps Toward Independence Program. Since Dr. Maser's professional research and technical skills lie in electron microscopy and the physical sciences, he will, at summer's end, also be charged with the supervision of our electron microscopy and radiation laboratories. Drs. Bruce Szamier and Anthony Liuzzi will continue their respective roles until September 1, 1977. This seems an appropriate place to acknowledge their contributions to the Laboratory.

Graduate and undergraduate instruction

The past year has seen only one significant change in the summer and January courses, which have continued unabated in the manner to which we have become accustomed—uniformly superior. Ivan Valiela of the Boston University Marine Program and John Teal of the Woods Hole Oceanographic Institution will be Co-Instructors-in-Charge of the Ecology course beginning in the summer of 1977. John and Ivan have been working together productively in research, and there is every reason to believe they will make a good team in teaching as well.

I am also pleased to be able to report three significant new departures in the offing. (1) Dr. Lucena J. Barth, who has contributed far more than anyone other than the Director can ever know, has accepted appointment as Director of Admissions, effective September 1, 1977. The several segments of our course and scholarship programs have each been done well, but in several different offices; consequently coordination and, all too often, timely promotion have been lacking. We are especially grateful for Florence Butz's past herculean efforts. (2) In keeping with our policy of encouraging colleges and universities to offer courses for which our facilities are uncommonly well suited, the Schools of Veterinary Medicine of Cornell University and the University of Pennsylvania together will offer a new course in Marine Veterinary Medicine ("Aquavets") in the late spring, 1977. The Schools will be joined in this effort by MBL and BUMP and by the Woods Hole Oceanographic Institution and the NOAA Northeast Fisheries Center. (3) Finally, to our "standard" January offerings (Behavior, Developmental Biology, Ecology, Neurobiology), we will add in 1978 another new course, Comparative Pathology of Marine Invertebrates, with a stellar cast headed by Frederik Bang and including Austin Farley, Jack Levin, Jack Marchalonis, Robert Prendergast and Carol Reinisch.

Year-round research

Six years ago the Laboratory took the first steps in a long-range program designed to augment and reinforce its capability for serving the Nation as a year-round center of advanced study and research in the genetics, development, neurobiology and ecology of marine organisms. The Laboratory's Officers and Trustees developed a comprehensive plan encompassing three closely related new programs, in ecology, neurobiology and the genetics of marine organisms, respectively. These fields were selected not only in recognition of the need to advance basic science in each of them, but also to improve their usefulness in the management of basic resources.

Two of these new initiatives are already well underway, *The Ecosystems Center*, under the direction of George Woodwell, and a series of programs in neurobiology and behavior in the Laboratory of Sensory Physiology and Laboratory of Biophysics of the National Institute of Neurological and Communicative Disorders and Stroke, headed by E. F. MacNichol, Jr. and W. J. Adelman, Jr.

The Laboratory now proposes to establish a research and training program in the genetics, development, pathobiology and pharmacology of marine organisms, and to develop a *Marine Resources Center*, which will not only provide laboratory space offering new research opportunities in these emerging fields, but will also make available expanded facilities for the collection and cultivation of marine organisms, responding to the demand for increasingly sophisticated research throughout the Nation.

The program will provide a vital underpinning for the technologies of aquaculture and mariculture. So much is written about "food from the sea" that we are inclined to think of it as a *fait accompli*. As we reflect, however, we realize that the field of mariculture (if indeed it can be called a field), is, in terms of its development, at about the level of the agricultural sciences some four decades ago, before genetics had made a significant impact.

For example, we know next-to-nothing about disease resistance in marine animals and plants; consequently many attempts to cultivate them *en masse* are doomed, almost from the start. We need to know the genetic basis (and the pathology) of disease, especially in those forms with the potential to supply significant amounts of protein.

Although the plans for the physical setting of the new Center are just being developed, its intellectual components, the scientists themselves, have already begun to interact. Throughout its history, the Laboratory has relied heavily on the catalysis provided by young minds. The development of the Marine Resources Center is no exception.

There has been a surprising concatenation of year-round investigators with related interests: Daniel Alkon and the able mariculturist, June Harrigan, in the Laboratory of Biophysics; Fred Lang of BUMP; James Oschman and Betty Wall; Ray Stephens, Kenneth Edds and Lewis Tilney in cell motility; and Judith Grassle and her colleagues in genetics. Although this array can hardly be said to be a "group," they make a significant nucleus.

The library

Our dialogue with the Woods Hole Oceanographic Institution, looking toward the establishment of a working-financial partnership in the operation of the Library, continues. I have invited Keith Porter to continue to represent the Laboratory in these discussions, as Chairman of an *ad hoc* organizational committee which includes Garland Allen, Clay Armstrong, Frederik Bang, John Buck, Harlyn Halvorson and David Shepro. Several drafts of an agreement have been completed, with the most recent being reviewed by the committee at this writing.

Gifford succeeds Robinson as Chairman

At their winter meeting in Woods Hole on February 11, the Trustees elected Dr. Prosser Gifford, Professor of History and Dean of the Faculty at Amherst College, as their Chairman to succeed Dr. Denis Robinson, who has served with distinction since 1971. Dr. Gifford, who was first elected to the MBL Board in 1975, has long taken an active interest in the Woods Hole scientific community, being deeply involved also in the Corporation of the Woods Hole Oceanographic Institution.

Both Dean Gifford and I spoke of Robinson's legacy of excellence. Like his predecessor, Gerard Swope, Jr., Dr. Robinson gained the affection and respect of the entire community for his statesmanship in creating at MBL an environment for year-round research and teaching destined to match the Laboratory's renowned summer programs.

Regrettably, Dr. Robinson's responsibilities as Chairman of the Board of High Voltage Engineering prevented him from attending the meeting. However, the Robinsons expect to be back at their home on Gosnold Road, Woods Hole, again this coming summer.

Honors

I am pleased to report that three members of the Corporation were elected to the National Academy of Sciences: Roderick Clayton, Aron Moscona and Ruth Sager.

1. The Staff

ECOLOGY

I. Instructors

Frederick E. Smith, Harvard University, director of course

George M. Woodwell, The Ecosystems Center, associate director of course
Daniel B. Botkin, The Ecosystems Center
Charles A. S. Hall, Cornell University and The Ecosystems Center
John E. Hobbie, The Ecosystems Center
Edgar Lemon, Cornell University
Kenneth H. Mann, Dalhousie University, Canada
Jerry M. Melillo, The Ecosystems Center
Theodore Packard, Bigelow Laboratory, Booth Bay Harbor, Maine
Mario M. Pamatmat, Auburn University
Bruce J. Peterson, The Ecosystems Center

II. Assistants

Timothy E. Wood, The Ecosystems Center
John V. Helfrich, The Ecosystems Center
James T. Morris, The Ecosystems Center
John F. Muratore, Yale University
David W. Juers, The Ecosystems Center

III. Special Lecturers

Thomas K. Duncan, Boston University
Robert R. Guillard, Woods Hole Oceanographic Institution
Thomas E. Lovejoy, World Wildlife Fund
Mary E. Nicotri, Woods Hole Oceanographic Institution
Howard L. Sanders, Woods Hole Oceanographic Institution
Wesley N. Tiffney, University of Massachusetts, Nantucket Field Station
Ivan Valiela, Boston University
Robert H. Whittaker, Cornell University

IV. Lectures

F. E. Smith	An introduction to ecology
G. M. Woodwell	The metabolism of ecosystems
H. L. Sanders	Benthic ecology
D. B. Botkin	Gas exchange measurements
H. L. Sanders	Community analysis
F. E. Smith	Problems with trophic levels
F. E. Smith	Logistics and chemostats
T. E. Wood	Dimension analysis
W. N. Tiffney	Sand dunes and cedar swamps
D. B. Botkin	Succession
I. Valiela	Salt marsh ecosystems
R. R. Guillard	Phytoplankton ecology
T. K. Duncan	Rocky intertidal ecosystems
F. E. Smith	Diversity-stability hypotheses
F. E. Smith	Density dependence in ecosystems
F. E. Smith	Spatial heterogeneity
D. B. Botkin	Spruce-moose interactions on Isle Royale
D. B. Botkin	Stability of ecosystems
C. A. S. Hall	Measuring aquatic ecosystem respiration
C. A. S. Hall	New Hope Creek: a study of ecosystem behavior
C. A. S. Hall	Ecosystem view of salmon life histories

G. M. Woodwell	Energy and carbon flow in land systems
J. E. Hobbie	Energy and carbon flow in a tundra pond
J. E. Hobbie	Primary production in aquatic systems
J. E. Hobbie	Bacteria in ecosystems
J. E. Hobbie	Respiration of plankton by long-term measurements of O_2 and CO_2 change
J. E. Hobbie	Respiration of bacteria and alga using ^{14}C techniques
T. Packard	Respiration of plankton by measuring the electron transport system
C. A. S. Hall	Respiration and energy flow in marsh-estuary ecosystems
J. Day	Estuarine ecology of Louisiana
F. E. Smith	Feeding links in models
F. E. Smith	Behavior of food-chain models
J. M. Melillo	Nutrient cycling in northern hardwoods
J. M. Melillo	Nitrogen cycling in forest ecosystems
M. M. Pamatmat	Introduction to benthic respiration
M. M. Pamatmat	Benthic metabolism
B. J. Peterson	Phosphorus cycling in aquatic ecosystems
D. B. Botkin	Contrasts of African and N. American ecosystems
G. M. Woodwell	Nutrient budgets of an estuarine marsh
M. E. Nicotri	Rocky-intertidal plant-herbivore interactions
K. H. Mann	Destruction of kelp beds by sea urchins: a cycle or an irreversible degradation?
K. H. Mann	Kelp bed ecosystems
K. H. Mann	Herbivores and detritivores in coastal waters
F. E. Smith	Natural selection in ecosystems
F. E. Smith	Ecosystem evolution
E. Lemon	Tropical rain forests: CO_2 exchange by shoestring
E. Lemon	CO_2 flux techniques
F. E. Smith	Planets as environments
G. M. Woodwell	Energy and the world carbon budget
G. M. Woodwell	Biotic impoverishment
R. H. Whittaker	Community structure, I and II
R. H. Whittaker	Indirect ordination and community evolution, I and II
T. E. Lovejoy	Endangered species
C. A. S. Hall	Modeling: philosophy
C. A. S. Hall	Modeling: basic terms and principles
J. T. Morris	Modeling: introduction to FOCAL
C. A. S. Hall	Flow diagrams
C. A. S. Hall	Fundamental relational concepts
J. T. Morris	Introduction to programming
F. E. Smith	Human ecology—some views
F. E. Smith	Urban ecology

EMBRYOLOGY

I. Instructors

Tom Humphreys, University of Hawaii, Pacific Biomedical Research Center, co-director of course

David Epel, University of California, San Diego, Scripps Institution of Oceanography, co-director of course

Laurence H. Kedes, Stanford School of Medicine

Michael Rosbash, Brandeis University
Barbara A. Hamkalo, University of California, Irvine
Joan V. Ruderman, Massachusetts Institute of Technology

II. Staff Associate

Linda S. Fregien, University of Hawaii

III. Assistants

Roger Duncan, University of Hawaii
Kenneth C. Kleene, University of Hawaii

IV. Lectures

J. D. Ebert	History of embryology at Woods Hole
G. Stent	Information in embryos
D. Epel	The role of Ca^{++}, Na^{+}, and K^{+} in the activation of sea urchin eggs
T. Humphreys	Activation of maternal mRNA
D. Epel	Echinoid eggs and embryos in developmental studies
J. V. Ruderman	*In vitro* translational analysis of mRNA in sea urchin and amphibian early development
J. Morrill	Gastropod eggs and embryos in developmental studies
T. Humphreys	RNA synthesis in sea urchin embryos
J. R. Whittaker	Ascidian eggs and embryos in developmental studies
R. A. Firtel	Gene activity during *Dictyostelium* development
B. P. Brandhorst	Two-dimensional gel electrophoretic analysis of protein in early sea urchin embryos
L. H. Kedes	Expression of histone genes during cleavage in sea urchin embryos
J. M. Arnold	Squid eggs and embryos in developmental studies
L. H. Kedes	Cloning and organization of the sea urchin histone genes
T. Humphreys	Nuclear and mRNA metabolism during sea urchin development
M. Rosbash	Kinetic considerations of molecular hybridization
M. Nemer	Nuclear and messenger RNA classes in sea urchin embryos
B. A. Hamkalo	Structure of chromosomes and chromatin
B. A. Hamkalo	Visualization of transcription
M. Rosbash	Evolutionary relatedness of mRNA and non-mRNA sequences in different species
J. Paul	Complexity of poly(A) mRNA and nuclear RNA in mouse friend cells
R. Grainger	Measurement of synthesis and turnover of RNA by density labeling
K. Van Holde	Properties of nucleosomes
J. Paul	Chromatin transcription and reconstruction
R. Roeder	Structure and function of eudaryotic RNA polymerases
R. Roeder	Transcription of eukaryotic genes *in vitro*
J. C. Hall	Fate mapping and the analysis of behavioral and developmental mutants in *Drosophila*
K. Illmensee	Developmental plasticity of mouse teratocarcinoma cells
B. E. Roberts	A direct method for locating protein coding regions within eukaryotic DNA

E. Davidson	Sequence organization and gene regulation in animals
E. Davidson	Gene activity during sea urchin development
R. L. White	R-loops, gene mapping, and gene selection

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. Instructors

Michael J. Greenberg, Florida State University, director of course
Brian L. Bayne, Institute for Environmental Research, Plymouth, U. K.
Stephen H. Bishop, Baylor College of Medicine, Houston
John H. Crowe, University of California, Davis
Rosevelt L. Pardy, University of California, Irvine
Sidney K. Pierce, Jr., University of Maryland
David J. Prior, University of Kentucky
Thomas J. M. Schopf, University of Chicago

II. Consultants

F. A. Brown, Jr., Northwestern University
C. Ladd Prosser, University of Illinois
Alfred C. Redfield, Woods Hole Oceanographic Institution
W. D. Russell-Hunter, Syracuse University
James Case, University of California, Santa Barbara
Robert K. Josephson, University of California, Irvine

III. Assistants

Roy White, University of Illinois
Wendy Wiltse, University of Massachusetts Marine Station
Steven O'Dell, University of California, Davis

IV. Secretary

Tonicia M. Smith, Marine Biological Laboratory

V. Special Lecturers

Harold L. Atwood, University of Toronto, Canada
Thomas Eisner, Cornell University
Esther M. Goudsmit, Oakland University, Michigan
Kjell Johansen, University of Aarhus, Denmark
Gilbert Y. Kennedy, Sheffield University, U. K.
Frederick Lang, Boston University Marine Program
Charlotte P. Mangum, College of William and Mary
Linda H. Mantel, City College, The City University of New York
Betty J. Wall, Marine Biological Laboratory
Peter H. Wiebe, Woods Hole Oceanographic Institution

VI. Lectures

M. J. Greenberg	Introductory notes
T. J. M. Schopf	Marine speciation: genetic and morphological differentiation

T. J. M. Schopf	The Cape Cod environment
T. J. M. Schopf	Marine faunal diversity: steady-state or an upward spiral?
T. J. M. Schopf	Marine faunal extinctions: crises in the history of life
S. K. Pierce	Salinity, osmosis, diffusion and, briefly, membrane structure
S. K. Pierce	Mechanisms of salinity tolerance: cell volume regulation
M. J. Greenberg	The distribution of animals with salinity: the horohalinicum as a physiological island
L. H. Mantel	Mechanisms of ionic regulation
L. H. Mantel	Hormonal control of ion regulation
L. H. Mantel	Adaptations of decapod crustaceans to various habitats
S. K. Pierce	The pause that refreshes: water balance through excretion
M. J. Greenberg	Circulation: volume, pressure and flow
M. J. Greenberg	Circulation: compartmentalization and filtration
J. H. Crowe	Transport of free amino acids by marine invertebrates
S. H. Bishop	Ammonia: formation, utilization, excretion and physiological role
S. H. Bishop	Control of amino acid pool sizes
F. Lang	Fight or flight in the lobster: growth and development of the claws and giant axons
S. H. Bishop	Nitrogen "detoxification"
S. H. Bishop	Phosphogens and the central role of arginine
J. H. Crowe	Anhydrobiosis: life without water
B. J. Wall	Water and ion regulation in the insect rectum
J. H. Crowe	Life in xeric environments
B. L. Bayne	Gas exchange theory with examples of various types of gas exchangers in marine invertebrates
P. H. Wiebe	North Atlantic cold core rings and their effect on the distribution, abundance and physiology of euphausids
M. J. Greenberg	Pigments, respiratory, invertebrate, four (4)
B. L. Bayne	Oxygen consumption related to weight and to temperature
B. L. Bayne	Oxygen consumption related to pO_2, aerial exposure and salinity
B. L. Bayne	Oxygen consumption related to ration; ecological aspects of gas exchange
B. L. Bayne	Heart rate, and control of gas exchange
R. L. Pardy	Fuels and metabolism
E. M. Goudsmit	Neurosecretory control of reproduction in the land snail, *Helix pomatia:* regulation of galactogen and glycogen synthesis
R. L. Pardy	Animal energetics
R. L. Pardy	Cellular aspects of algal symbiosis
R. L. Pardy	Metabolic/energetic aspects of symbiosis
C. P. Mangum	How oxygen transport systems work in unstable environments
D. J. Prior	Neural and hormonal control systems
D. J. Prior	Neural control of behavior
H. L. Atwood	Crustacean neuromuscular physiology, with some ecological afterthoughts
D. J. Prior	Integration—sites for modification of neural output
D. J. Prior	Adaptation of nervous systems to environmental pressures
K. Johansen	How animals obtain oxygen when there is little of it (a fishy story)

G. Y. Kennedy	Porphyrins in invertebrates
T. Eisner	Social defense strategy in a termite
T. Eisner	A beetle's approach to survival: sticky feet and a fecal parasol
T. Eisner	Insect and plant co-evolution: a chemical approach (with implications to humans?)

EXPERIMENTAL MARINE BOTANY

(Comparative Biology and Biochemistry of Algae)

I. Instructors

Jerome A. Schiff, Brandeis University, director of course
David Mauzerall, The Rockefeller University
Robert F. Troxler, Boston University
Richard J. Ellis, Bucknell University, laboratory instructor
James R. Sears, Southeastern Massachusetts University, laboratory instructor

II. Consultants

Robert L. Guillard, Woods Hole Oceanographic Institution
Frank A. Loewus, Washington State University
Ralph S. Quatrano, Oregon State University

III. Staff Associates

Hans Gaffron, University of Florida
Gary Harris, Wellesley College

IV. Assistants

Sherry Sass, Southeastern Massachusetts University
Rosemarie Vonusa

V. Lectures

J. A. Schiff	Chemical phase of evolution: biogeochemistry
J. A. Schiff	The appearance of oxygen
J. A. Schiff	Evolution of procaryotes
R. J. Ellis	The dinoflagellate nucleus: a link in the evolution of eucaryotes?
J. A. Schiff	Evolution of eucaryotes and organelles
J. A. Schiff	Evolution of life cycles
J. A. Schiff	Nutritional cycles
M. L-S. Tsang	Metabolism of nitrogen and sulfur
U. Goodenough	Mating interactions in *Chlamydomonas*
R. J. Ellis	Heme and chlorophyll biosynthesis: early steps
R. F. Troxler	Heme and chlorophyll biosynthesis: later steps
R. F. Troxler	Biosynthesis of open chain tetrapyrroles
H. W. Siegelman	Carotenochlorophyll protein of the dinoflagellates
D. Mauzerall	Chromatic adaptation
J. R. Sears	Vertical distribution of benthic algae
D. Mauzerall	Photochemistry of photosynthesis

J. Gibson	Photosynthetic electron transport
S. Cohen	(with R. J. Ellis, G. Harris, A. Holowinsky, D. Mauzerall, J. A. Schiff, R. F. Troxler and others: informal discussion on organelle structure, development, origin and evolution)
A. I. Stern	Photophosphorylation
H. Gaffron	Catalytic effects of CO_2 on the Hill Reaction
G. Harris	Ribulose bisphosphate carboxylase/oxygenase
J. Waterbury	Biology of the blue green algae
E. E. Conn	Cyanogenesis in plants
S. Cohen	Viruses of blue green algae (cyanophages)
R. D. Simon	Cyanophycin granules
N. Lazaroff	Photocontrol of morphogenesis in blue green algae
R. E. Stephens	Microtubules in plant development
R. Jones	Turnover of macromolecules during the cell cycle of *Chlamydomonas*
R. L. Guillard	Concepts involved in the hypothesis that environmental factors influence the distribution of phytoplankton species
M. Gibbs	Photoproduction of hydrogen
J. R. Sears	Chlorophyta I, II
A. Coleman	Chlorophyta III (colonial green flagellates)
R. J. Ellis and J. R. Sears	Chlorophyta IV
J. A. Schiff	Euglenophyta
R. J. Ellis	Pigment synthesis in Euglenophyta
J. R. Sears	Phaeophyta I, II, III
H. W. Siegelman	High pressure liquid chromatography
R. J. Ellis	Pigment methodology
R. J. Ellis and R. F. Troxler	Phycobilin methodology
J. R. Sears	Algal productivity and pigments
R. J. Ellis	Physiology, reproduction and development
R. J. Ellis	Phototaxis and chemotaxis
R. J. Ellis	Osmotic relations and ion absorption
J. R. Sears	Rhodophyta I, II, III
R. J. Ellis	Xanthophyta
J. R. Sears	Chrysophyta
R. J. Ellis	Heterotrophy, mixotrophy and obligate autotrophy
L. Murphy	Algal genetics, phytoplankton ecology

NEUROBIOLOGY

I. Instructors

Edward A. Kravitz, Harvard Medical School, co-director of course
Antony O. W. Stretton, Wisconsin University, co-director of course
Philippa Claude, Harvard Medical School
Gerald D. Fischbach, Harvard Medical School
Edwin J. Furshpan, Harvard Medical School
John Heuser, University of California, San Francisco
Paul O'Lague, Harvard Medical School

David D. Potter, Harvard Medical School
Thomas S. Reese, National Institutes of Health

II. Staff Associates

Barbara A. Battelle, Harvard Medical School
Louise Brams, National Institutes of Health
Margaret Livingstone, Harvard Medical School
Barbara Reese, National Institutes of Health
Lee Rubin, Harvard Medical School
William Woodward, Harvard Medical School

III. Assistants

Judy Donmoyer, Wisconsin University
Ruth Siegel, Harvard Medical School
Quan Yang Duh, National Institutes of Health

IV. Special Lecturers

C. Anderson, University College, London, U. K.
Clay M. Armstrong, University of Pennsylvania
M. V. L. Bennett, Albert Einstein College of Medicine
Lawrence B. Cohen, Yale University
Michael D. Calahan, University of Pennsylvania
Robert L. DeHaan, Emory University
Kathy Dunlop, University of California, Los Angeles
Alan Finkelstein, Albert Einstein College of Medicine
Bertil Hille, University of Washington School of Medicine
William T. Keeton, Cornell University
Yoram Lass, Tel Aviv University, Israel
Peter Marler, The Rockefeller University
Joanna B. Olmstead, University of Rochester
Jim Oschman, Marine Biological Laboratory
Harvey B. Pollard, National Institutes of Health
Keith R. Porter, MBL and University of Colorado, Boulder
Jack Rosenbluth, New York University
Stephen Smith, University of Washington
Charles F. Stevens, Yale University
Ann E. Stuart, Harvard Medical School
Asher E. Treat, American Museum of Natural History
Betty Wall, Marine Biological Laboratory
Edward O. Wilson, Harvard University

V. Lectures

C. Anderson	Conductance and gating kinetics of single postsynaptic ionic channels
G. D. Fischbach	Tissue culture technique
B. Hille	Structure and function of sodium channels in axon membranes
C. M. Armstrong	Gating currents from axons
C. F. Stevens	Analysis of neurotransmitter-induced conductance increases

G. D. Fischbach	Development of electrical excitability
J. B. Olmstead	Microtubules and the development of cell shape
G. D. Fischbach	Development of chemical excitability
G. D. Fischbach	Synapse formation
Y. Lass	Temperature dependence of ACh noise in cultured myoballs
E. Frank	Reinnervation of peripheral ganglia and muscles
K. Dunlop	Calcium spikes in paramecia
K. R. Porter	Scanning microscopy and some biological applications
E. O. Wilson	The organization of insect societies
P. Marler	The development of stimulus recognition systems in animals
W. T. Keeton	Bird navigation: new developments in an old mystery
T. S. Reese	General cytological features of nervous systems
J. Oschman and B. Wall	Calcium, phosphate and membranes
T. S. Reese	Nonsynaptic junctions in the nervous system
J. E. Heuser	Structure of synapses: the presynaptic membrane, I and II
J. Rosenbluth	Intramembranous particle distribution at the node of Ranvier and adjacent axolemma in myelinated axons of the frog brain
T. S. Reese	Structure of synapses: the postsynaptic membrane
P. Claude	Axoplasmic flow
M. V. L. Bennett	Correlations between electrophysiological properties and intramembranous particles
J. E. Heuser	Chromatolysis
A. Finkelstein	The interaction of black widow spider venom with lipid bilayer membranes
P. Claude	Development of synapses: cytological aspects
A. E. Stuart	Initial stages in transmission of visual signals in the nervous system of the giant barnacle
H. B. Pollard	Secretory vesicles, cyclic AMP and transmembrane potentials
A. O. W. Stretton	Acetylcholine
A. O. W. Stretton	Catecholamines
E. A. Kravitz	GABA
E. A. Kravitz	Octopamine and other amines in lobsters: possible physiological roles
E. A. Kravitz	Central nervous system: I. Peptides
A. E. Treat	Who's who in a moth ear: a tale of two species
B. A. Battelle	Cyclic nucleotides
E. A. Kravitz	Central nervous system: II. Amines and modulation
L. B. Cohen	Optical measurements of neuron activity
M. D. Calahan	Pharmacological modification of sodium channels
S. Smith	Calcium ions and bursting pacemaker activity in molluscan neurons
R. L. DeHaan	Action currents in embryonic heart cells in culture

PHYSIOLOGY

I. Instructors

John J. Cebra, The Johns Hopkins University, director of course
Gary Ackers, University of Virginia

Dennis Barrett, University of Denver
Pien-chien Huang, The Johns Hopkins School of Hygiene and Public Health
Ru-chih C. Huang, The Johns Hopkins University
Jean Lindenmann, University of Zurich
Jerry B. Lingrel, University of Cincinnati College of Medicine
Joanna B. Olmsted, University of Rochester
Thomas D. Pollard, Harvard Medical School
Dennis A. Powers, The Johns Hopkins University
Robert A. Prendergast, The Johns Hopkins School of Medicine
Roger B. Taylor, University of Bristol, U. K.
Gerald Weissmann, New York University School of Medicine

II. Consultants

Gerald Cole, The Johns Hopkins School of Hygiene and Public Health
Philip B. Dunham, Syracuse University
Daniel Goodenough, Harvard Medical School
William Marzluff, Florida State University
Carol Reinisch, Harvard Medical School

III. Staff Associates

Paula Barbiaz, Syracuse University
Judith A. Cebra, The Johns Hopkins University
Rebecca Emmons, The Johns Hopkins University
Harold Drabkin, Wesleyan University
Marcus Fechheimer, The Johns Hopkins University
Alan Israel, New York University School of Medicine

IV. Assistants

Martha B. Barrett, University of Colorado
Patrick Galvin, University of Colorado

V. Special Lecturers

R. D. Allen, University of New Hampshire
Shinya Inoué, University of Pennsylvania
Norman Klinman, University of Pennsylvania
Robert Kretzinger, University of Virginia
Salvador E. Luria, Massachusetts Institute of Technology
Joel Rosenbaum, Yale University
Andrew G. Szent-Györgyi, Brandeis University
William Trager, Rockefeller University
Daniel Kiehart, University of Pennsylvania
Margaret A. Leong, University of California, Davis
Paul Mied, The Johns Hopkins University
Mark M. Mooseker, Harvard Medical School
Jeffrey Moshier, The Johns Hopkins University
Louise Ramm, University of Virginia
Glenn Smith, The Johns Hopkins University
James Stanchfield, Dartmouth College
Henry Steinberg, The Johns Hopkins University

Susan J. Tucker, The Johns Hopkins University
Patricia P. Watson, The Johns Hopkins University

VI. Lectures

D. Goodenough	The structure of biomembranes: historical perspectives
D. Goodenough	The structure of biomembranes: current theories
D. Goodenough	Structure and function of some intercellular junctions
G. Weissmann	Lysosomes and the vacuolar system
G. Weissmann	Liposomes as model membrane systems
P. Dunham	Enzymes of the cell membrane: the ion pump
J. Cebra	Antibodies: relationships of structural features to their various activities and specificities
R. B. Taylor	The antibody response: cellular and humoral aspects
R. A. Prendergast	Cell-mediated immune responses
R. B. Taylor	Control of the immune response: specific regulation of a complex system
J. Cebra	B-lymphocyte ontogeny: the development of the secretory immunoglobulin-A system
N. Klinman	Expression of the B-cell repertoire
R. B. Taylor	Recognition of self *vs.* nonself
R. A. Prendergast	Sea star coelomocyte protein and modulation of the vertebrate immune response
G. Cole	Host responses to virus infections
G. Weissmann	Lysosomes and immune reactions
J. Lingrel	Messenger RNA: general structural features and their relation to function; mRNA turnover
J. Lingrel	Is there a precursor to mRNA?
P. C. Huang	Cell proliferation, DNA replication and repair
J. Lindenmann	T-cell receptors for alloantigens
P. C. Huang	Genome and primary transcripts; signals in the base sequences
P. C. Huang	Chromosomal nucleoproteins: structure and function
D. Barrett	Transcriptional control in eukaryotic development
D. Barrett	Translational control in eukaryotic development
J. Lingrel	The number of globin genes: genetic studies and the use of molecular probes
R. Huang	Tissue-specific mRNAs and proteins of highly-differentiated systems
R. Huang	Specificities of chromatin templates and RNA polymerases
R. Huang	Assay systems for RNA synthesis and transcription *in vitro* of specific genes
D. Powers	Hemoglobin: a protein for all seasons
D. Powers	Genes and demes: a biochemical approach to the study of genetic variability
G. Ackers	How do macromolecules interact?
G. Ackers	What do hemoglobin subunits do to each other when they get together?
J. Olmsted	Microtubule structure and chemistry
J. Olmsted	Microtubule assembly
S. Inoué	Mitosis
T. Pollard	Contractile protein biochemistry and structure

T. Pollard	Force-generating mechanisms in muscle and nonmuscle cells
A. Szent-Györgyi	Mechanisms regulating contractile proteins
M. Mooseker	Model motile systems
R. D. Allen	Cellular aspects of motility
T. Pollard	Cytoplasmic matrix
T. Pollard	Primitive motile systems
J. Olmsted	Microtubules and motility
J. Rosenbaum	Flagellar assembly
P. Dunham	Na-K transport in erythrocytes of sheep and goats: antibody-induced alterations
W. Marzluff	Synthesis and maturation of RNA: an *in vitro* approach
S. Luria	Uses of membrane energy
J. Lindenmann	Host antigens in enveloped viruses: I and II
W. Trager	Human malaria parasites in continuous culture
R. Kretsinger	X-ray crystallography of calcium-binding proteins

JANUARY COURSES 1976

BEHAVIOR

(*Offered Jointly by Boston University Marine Program and the Marine Biological Laboratory*)

I. Instructor

Robert L. Jeanne, Boston University, director of course

II. Assistant

Stuart Jacobson, Boston University Marine Program

III. Special Lecturers

Jelle Atema, Boston University
Stanley Fox, Boston University
Donald Griffin, The Rockefeller University
Jeremy Hatch, University of Massachusetts, Boston
Adrianus Kalmijn, Woods Hole Oceanographic Institution
George Michel, Boston University
Celia Moore, University of Massachusetts, Boston
Bori Olla, Middle Atlantic Coastal Fisheries Center
Alastair Stuart, University of Massachusetts, Amherst

IV. Lectures

R. L. Jeanne	Introduction to the course; basic statistical concepts
J. Atema	Sensory physiology and behavior
J. Atema	Structure and function of chemoreceptors and mechanoreceptors
J. Atema	Evolution of chemoreception
A. Kalmijn	Electroreception in object and prey detection
A. Kalmijn	Orientation and navigation in electric and magnetic fields
R. L. Jeanne	Endogenous *vs.* exogenous control of biological rhythms
G. Michel	The role of learning in behavior

C. Moore	Constraints on learning
C. Moore	Hormones and experience in ring dove reproduction
G. Michel, C. Moore, J. Atema, and R. L. Jeanne	Discussion: Description and analysis of behavior
J. Atema	Mating behavior in the lobster
R. L. Jeanne	Introduction to orientation
D. Griffin	Orientation behavior in bats and birds
D. Griffin	Information transfer in bee dances
D. Griffin	The question of mental continuity
D. Griffin and A. Kalmijn	The evidence for sensitivity to the earth's magnetic field
R. L. Jeanne	Introduction to sociobiology
B. Olla	Natural history and behavior of marine fishes: the importance of defining habits under natural conditions
B. Olla	Behavioral measures of environmental stress
B. Olla	Social interactions of marine fishes: schooling and feeding facilitation
B. Olla	Film session: feeding and schooling in pelagic fish; aggression and territoriality in demersal fish
G. Michel	Primate socialization
G. Michel	Kidnaping and care-taking in primates
R. L. Jeanne	Introduction to communication
R. L. Jeanne	Information theory and the analysis of communication
J. Atema	Social behavior and communication in catfish
S. Fox	Territory, aggression, and dispersal
S. Fox	Natural selection among behavioral phenotypes of the lizard *Uta stansburiana*
J. Hatch	Bird song
J. Hatch	Seminar
A. Stuart	Social behavior of the termites
A. Stuart	Termite communication
R. L. Jeanne	Social behavior in the Hymenoptera
R. L. Jeanne	Adaptations of nest architecture in the social wasps
R. L. Jeanne	Altruism and group selection

DEVELOPMENTAL BIOLOGY

I. Instructors

Walter S. Vincent, University of Delaware, director of course
Louis E. DeLanney, Ithaca College
James D. Ebert, Carnegie Institution and Marine Biological Laboratory
Susan Gerbi, Brown University
Eve MacDonald, Wilson College
J. Richard Whittaker, Wistar Institute

II. Staff Associate

Stanley Krane, Fitchburg State College

III. Assistant

William D. Bates, University of Pennsylvania

IV. SPECIAL LECTURERS

EUGENE BELL, Massachusetts Institute of Technology
NOEL DE TERRA, Institute for Cancer Research
KENNETH T. EDDS, Marine Biological Laboratory
ELIZABETH HAY, Harvard University
ARTHUR G. HUMES, Boston University and Marine Biological Laboratory
W. D. MASSOVER, Brown University
S. MERYL ROSE, Woods Hole
RAYMOND E. STEPHENS, Brandeis University and Marine Biological Laboratory
GLADYS K. SCHLANGER, Cornell Medical College
EMILE ZUCKERKANDL, University of Delaware and Marine Biological Laboratory

V. LECTURES

W. S. VINCENT	Introduction to the Developmental Biology course
W. S. VINCENT	The egg as a lesson in cell biology
W. S. VINCENT	Ovary *vs.* testis: genetics *vs.* environment
W. S. VINCENT	Oocyte development: I. Cell organelles
W. S. VINCENT	Oocyte development: II. The germinal vesicle nucleus
W. S. VINCENT	Oogenesis, an exercise in future think
E. MACDONALD	Spermiogenesis
E. MACDONALD	Techniques of transmission and scanning electron microscopy
L. DELANNEY	Immune systems as a model for development: I, II, III
J. R. WHITTAKER	Cytoplasmic information in development: I. Mosaic development
J. R. WHITTAKER	Cytoplasmic information in development: II. Germ plasm and axiation factors
J. R. WHITTAKER	Cytoplasmic information in development: III. Nuclear-cytoplasmic interactions
J. R. WHITTAKER	Is differentiation reversible?
J. R. WHITTAKER	Developmental approaches to malignancy
N. DE TERRA	New ideas about cleavage
S. GERBI	Mitosis and meiosis
S. GERBI	DNA replication
S. GERBI	Chromosome structure: I. Chromatin
S. GERBI	Chromosome structure: II. Cytology
S. GERBI	Chromosome structure: III. and IV. Molecular architecture
S. GERBI	Evolution of satellite and ribosomal DNA's
J. D. EBERT	Interacting systems in development
J. D. EBERT	Ionic regulation of embryonic induction
S. M. ROSE	Regeneration in denervated limbs in amphibians
A. G. HUMES	Post-embryonic development of Copepoda
E. BELL	Genome utilization during differentiation
S. KRANE	Restriction enzymes
G. K. SCHLANGER	Development of chloroplast ribosomes
W. S. VINCENT	Rate control of gene expression
R. E. STEPHENS	Ciliogenesis
W. D. MASSOVER	New concepts of vitellogenesis
N. DE TERRA	Stentorian development
S. INOUÉ	Spicule morphogenesis

H. LODISH	Messenger RNA in slime mold development
H. O. HALVORSON	Sporulation as a developmental model
K. R. PORTER	Microtubules in cellular morphogenesis
S. GERBI	Evolution of satellite and ribosomal DNA's
J. D. EBERT	Birth defects, prospects for progress
K. T. EDDS	The role of microfilaments in sea urchin development
E. HAY	Tissue interaction in the developing cornea
E. ZUCKERKANDL	Programs of gene action and progressive evolution
E. BELL	Films on morphogenesis
A. SZENT-GYÖRGYI	New theories of cancer

ECOLOGY

I. INSTRUCTORS

GEORGE M. WOODWELL, The Ecosystems Center, Marine Biological Laboratory, director of course
DANIEL B. BOTKIN, The Ecosystems Center
CHARLES A. S. HALL, Cornell University, and The Ecosystems Center
BRUCE J. PETERSON, The Ecosystems Center

II. ASSISTANTS

TIMOTHY E. WOOD, The Ecosystems Center
PAUL A. STEUDLER, The Ecosystems Center
JOHN V. K. HELFRICH, The Ecosystems Center
RICHARD A. HOUGHTON, The Ecosystems Center
DAVID W. JUERS, The Ecosystems Center

III. SPECIAL LECTURERS

LYNN MARGULIS, Boston University
HOWARD L. SANDERS, Woods Hole Oceanographic Institution
KENNETH O. EMERY, Woods Hole Oceanographic Institution
JOHN M. TEAL, Woods Hole Oceanographic Institution
STEPHEN H. BERWICK, Yale University
EVILLE GORHAM, University of Minnesota
HOLGER W. JANNASCH, Woods Hole Oceanographic Institution
KENNETH H. MANN, Dalhousie University, Canada
MARILYN J. JORDAN, Cornell University
EDWARD O. WILSON, Harvard University
JERRY M. MELILLO, Yale University
IVAN VALIELA, Boston University
J. GUSTAVE SPETH, Natural Resources Defense Council
THOMAS COCHRAN, Natural Resources Defense Council
RICHARD C. HENNEMUTH, National Marine Fisheries
FREDERICK E. SMITH, Harvard University
EUGENE BELL, Massachusetts Institute of Technology
JOHN E. HOBBIE, The Ecosystems Center

IV. LECTURES

G. M. WOODWELL	Introduction to the MBL
G. M. WOODWELL	The biosphere: a set of interacting ecosystems

D. B. Botkin	The history of the earth: the evolution of the biosphere
L. Margulis	Microbial evolution and the precambrian environment
H. L. Sanders	Natural communities and evolutionary strategies, I and II
K. O. Emery	Continental drift
G. M. Woodwell	Climates of the world
G. M. Woodwell	Plant geography: the vegetation of the earth
J. M. Teal	Oceans of the world
C. A. S. Hall	Lakes of the world
D. B. Botkin	The earth as a thermodynamic system
G. M. Woodwell	Primary production and the metabolism of the earth
S. H. Berwick	Zoogeography: Southern Asia as a paradigm, I and II
C. A. S. Hall	Secondary productivity: aquatic, I and II
D. B. Botkin	Succession
D. B. Botkin	Secondary productivity: terrestrial
E. Gorham	Paleoecology, I and II
G. M. Woodwell	Basic ecology from worldwide pollution
G. M. Woodwell	Experimental eutrophication of terrestrial and aquatic ecosystems
B. J. Peterson	Nutrient limitation in freshwater systems
C. A. S. Hall	Nutrient limitation in saltwater systems
H. W. Jannasch	The microbial sulfur cycle
H. W. Jannasch	Heterotrophic microbial activities in the deep ocean
K. H. Mann	Primary production in marine systems
M. J. Jordan	Industrial pollution
T. E. Wood	Acid precipitation
E. O. Wilson	Sociobiology
J. M. Melillo	Nutrient cycles, I and II
I. Valiela	Nutrient cycling in a salt marsh
G. M. Woodwell	Ecosystems and world politics
D. B. Botkin	The stability of ecosystems, I
J. E. Hobbie	The stability of ecosystems, II
J. G. Speth and T. Cochran	Environment and law
J. G. Speth and T. Cochran	New forces of change
A. Szent-Gyorgyi	New directions in cancer research
R. C. Hennemuth	The future of fish, I and II
F. E. Smith	Darwinian selection among urban ecosystems
F. E. Smith	Urban ecosystems, I and II
E. Bell	The world food problems
G. M. Woodwell	Biotic impoverishment and the threshold dilemma

NEUROBIOLOGY

(*Offered jointly by Boston University Marine Program and the Marine Biological Laboratory*)

I. Instructors

Frederick Lang, Boston University Marine Program, director of course
Alan Fein, Marine Biological Laboratory
Edward F. MacNichol, Jr., Assistant director, Marine Biological Laboratory

II. Consultants

Frederick A. Dodge, The Rockefeller University
Stanely B. Kater, University of Iowa

III. Assistants

Walter J. Costello, Boston University
Daniel G. Gibson, Boston University

IV. Special Lecturers

William J. Adelman, National Institutes of Health
Daniel L. Alkon, National Institutes of Health
Jelle Atema, Boston University
Melvin J. Cohen, Yale University
Donald T. Frazier, University of Kentucky
Robert J. French, National Institutes of Health
Edward A. Kravitz, Harvard Medical School
Peter MacLeish, Harvard Medical School
Paul O'Bryan, Boston University School of Medicine

V. Lectures

E. F. MacNichol, Jr.	Electrophysiological techniques
A. Fein	Neuron doctrine—anatomy of the nervous system
A. Fein	Membrane structure
R. J. French	Bookkeeping for a cell—an ionic balance sheet
R. J. French	Ionic diffusion across liquid junctions and cell membranes
R. J. French	Criteria for independence and active transport
W. J. Adelman	Membrane potentials
W. J. Adelman	The nerve impulse
W. J. Adelman	Membrane currents
W. J. Adelman	Components of membrane conductance
W. J. Adelman	Channels and model systems
F. Lang	Synaptic transmission—the soups *vs.* the sparks
F. Lang	Ionic basis of synaptic potentials
F. Lang	Quantal release: excitation-secretion coupling
F. Lang	Vesicle hypothesis
F. Lang	Electrical synapses: facilitation and arthropod neuromuscular systems
A. Fein	Crustacean stretch receptor: Pacinian corpuscle
A. Fein	Invertebrate photoreceptors
A. Fein	Muscle spindles
J. Atema	Functions of taste and smell
M. Cohen	Factors influencing dendritic structure and function in identified central neurons
F. A. Dodge	A quantitative description of the vertebrate motor neuron from dendrite to terminal: I. Excitability and propagation of nerve impulses in myelinated nerve fibers; II. Excitability of spinal motor neurons
D. T. Frazier	Neural control of respiration in vertebrates
E. A. Kravitz	Octopamine neurons in lobster
S. B. Kater	Neural basis of feeding in the pulmonate snail *Heliosoma trivolvis*

P. O'Bryan	Vertebrate retina
P. MacLeish	Synapse formation between sympathetic neurons in culture
D. Alkon	Signal transformation with pairing of sensory stimuli

YEAR-IN-SCIENCE
1975–1976

I. Instructors

George M. Woodwell, The Ecosystems Center, Marine Biological Laboratory, director of course
Daniel B. Botkin, The Ecosystems Center
Charles A. S. Hall, Cornell University and The Ecosystems Center
John E. Hobbie (spring semester 1976), The Ecosystems Center
Bruce J. Peterson, The Ecosystems Center

II. Assistants

John V. K. Helfrich, The Ecosystems Center
Richard A. Houghton, The Ecosystems Center
David W. Juers, The Ecosystems Center
James T. Morris, The Ecosystems Center
Paul A. Steudler, The Ecosystems Center
Timothy E. Wood, The Ecosystems Center

III. Special Lecturers

Stephen H. Berwick, Yale University
Howard L. Sanders, Woods Hole Oceanographic Institution
Robert R. Parker, Commonwealth Scientific and Industrial Research Organization
Jerry M. Melillo, Yale University
John Milton, Thresholds, Washington, D. C.
Walter E. Westman, University of California
Myron L. Heinselman, U. S. Forest Service (retired)
John P. Holdren, University of California, Berkeley
Arturo Gomez-Pompa, Biological Institute, Mexico City
Jo-Ann P. Roskoski, Yale University
John D. Aber, Yale University
John Todd, The New Alchemy Institute, Falmouth, Massachusetts
Jawahar Tiwari, The Ecosystems Center
James Haney, University of New Hampshire

IV. Lectures

Fall 1975

G. M. Woodwell	The vegetation of the earth
S. H. Berwick	Zoogeography: Southern Asia as a paradigm
H. L. Sanders	Marine benthic diversity
R. R. Parker	A systems analysis of an ecosystem
L. S. Collins*	Trophic structure of ecosystems
B. F. Leon*	Succession I
R. D. Boone*	Succession II
G. M. Woodwell	Carbon metabolism/energy I

D. B. Botkin	The carbon budget II: effects of enhancement of atmospheric CO_2
J. M. Melillo	Nitrogen metabolism in ecosystems
B. F. Leon* and T. E. Wood	The world sulfur budget
J. Milton	Ecology and international development
J. Milton	Land use in the Himalayas
D. B. Botkin	Nutrient cycles: sodium in a temperate zone ecosystem
C. L. Van Dover*	The Redfield Hypothesis
M. L. Heinselmann	Fire in terrestrial communities
G. M. Woodwell	Ecological effects of toxic substances
W. E. Westman	Vegetation modification for fire control: a case study in Southern California chaparral
W. E. Westman	Resiliency in vegetation: studies in Australian coastal vegetation
W. E. Westman	Water pollution control legislation in the U. S. and Australia
R. A. Houghton	Design of ecosystems
R. A. Houghton	A ten-year record of atmospheric CO_2 in the northeast U. S

Spring 1976

R. A. Houghton	Carbon models: state of the art
L. S. Collins*	Effects of atmospheric CO_2 on climate
J. P. Holdren	Energy from fusion: how much difference will it make?
B. A. Pierce*	The ocean buffer system
J. Tiwari	Random differential equations and information theory approach to the modelling of ecosystems
R. M. Hurd*	The effects of CO_2 fertilization
D. W. Juers	Carbon components in sea water and techniques of measurement
J. Haney	Regulation of grazing by Cladocera
T. B. Brown*	Carbon on a geologic time scale
J. Todd	Designing contained food-producing ecosystems
T. E. Wood	Limits to production of CO_2
B. J. Peterson	Carbon budget for lakes
J. P. Roskoski	Nitrogen fixation in a forest ecosystem
C. L. Van Dover*	The detrital component of marine ecosystems
B. J. Peterson	Relationship between ^{14}C uptake and net particulate carbon production
J. D. Aber	Competition for light and nutrients in northern forests
R. A. Houghton	A single year model of the global carbon cycle
A. Gomez-Pompa	The effects of industrialization in Mexico
J. T. Morris and T. E. Wood	The consequences of varying parameters in the long-term carbon cycle model

THE LABORATORY STAFF

(*Including all whose services began or ended during the year.*)

Homer P. Smith, *General Manager*

Francis P. Bowles, *Coordinator of Research Services*

A. Robert Gunning, *Superintendent, Buildings and Grounds*

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* Year-in-Science student seminar.

Teresa M. Braun, *Assistant Editor, The Biological Bulletin*

Edward G. Casey, *Controller*

Jane Fessenden, *Librarian*

Jim A. Hancock, *Manager, Research Services*

Lewis M. Lawday, *Assistant Manager, Supply Department*

John J. Valois, *Manager, Supply Department*

DIRECTOR'S OFFICE

Marie B. Abbott, *Curator, Gray Museum*

Lucena J. Barth

Pamela J. Foose

Edward F. MacNichol, Jr., *Assistant Director for Research Services*

Maureen M. Morris

Charles A. Ossola, *Assistant Director for Finance and Development*

George M. Woodwell, *Assistant Director for Education and Director of The Ecosystems Center*

GENERAL MANAGER'S OFFICE

Florence S. Butz

Elaine C. Crocker

Agnes L. Geggatt

CONTROLLER'S OFFICE

Doris Carmo

Doris C. Davis

Shirley J. Delisle

Nancy L. Ellis

Joan E. Howard

Lorraine A. Ruddick

Mary R. Tavares

LIBRARY

Judith A. Ashmore

Rebecca J. Bailey

Nancy M. Capuano

David J. Fitzgerald

Charlotte F. Goudreau

Joan H. Grice

Catherine Herrity

E. Lenora Joseph

Holly E. Karalekas

Theresa K. McKee

M. Ann White

BUILDINGS AND GROUNDS

Lee E. Bourgoin

John V. Day

Manuel P. Dutra

Glenn R. Enos

Charles K. Fuglister

Elizabeth J. Geggatt

Richard E. Geggatt, Jr.

Robert J. Golder

Robert L. Grosch

Robert A. Hinz

Thomas N. Kleindinst

Elisabeth Kuil

Donald B. Lehy

Ralph H. Lewis

William M. Lochhead

Daniel Loewus

Richard C. Lovering

Alan G. Lunn

John B. MacLeod

John E. Maurer

Stephen A. Mills

Glenn I. Shear

Frederick E. Thrasher

Frederick E. Ward

Ralph D. Whitman

William Whittaker

DEPARTMENT OF RESEARCH SERVICES

Thomas R. Anthony
Franklin D. Barnes
John Barnes
Julie A. Caissie
Gail Cavanaugh
Linda M. Golder
Kathryn L. Helfrich
Lowell V. Martin
Joan A. Peters-Gilmartin
Frank E. Sylvia

SUPPLY DEPARTMENT

Edward G. Enos, Jr.
Joyce B. Enos
Robert M. Hebden
Mark W. Munson
John H. Ryther, Jr.
A. Dickson Smith
Eugene A. Tassinari
Bruno F. Trapasso
John M. Varao
Frederick W. von Arx

2. Investigators; Research Fellowships; Students

Independent Investigators, 1976

Ackers, Gary K., Professor and Chairman of Biophysics, University of Virginia
Adelman, William J., Jr., Chief, Laboratory of Biophysics, NINCDS, National Institutes of Health
Akaike, Tadashi, Visiting Associate, NINCDS, National Institutes of Health
Alkon, Daniel L., Research Medical Officer, NINCDS, National Institutes of Health
Allen, Nina Strömgren, Assistant Professor, Dartmouth College
Allen, Robert D., Professor and Chairman of Biology, Dartmouth College
Almers, Wolfhard, Assistant Professor of Physiology and Biophysics, University of Washington
Armstrong, Clay M., Professor of Physiology, University of Pennsylvania
Armstrong, Peter B., Associate Professor of Zoology, University of California, Davis
Arnold, John M., Professor, University of Hawaii
Arnold, Michael, Investigator, National Foundation for Cancer Research
Atwood, Harold L., Professor of Zoology, University of Toronto, Canada
Ball, Eric G., Professor Emeritus, Harvard Medical School
Barlow, Robert B., Jr., Associate Professor, Institute for Sensory Research, Syracuse University
Battelle, Barbara-Anne, Research Fellow, Harvard Medical School
Bauer, G. Eric, Associate Professor of Anatomy, University of Minnesota
Baumgold, Jesse, Staff Fellow, NIMH, National Institutes Health
Bayne, Brian L., Principal Scientific Officer, Institute for Marine Environmental Research, United Kingdom
Beeson, Karl W., Research Associate, Columbia University
Begenisich, Ted, Assistant Professor of Physiology, University of Rochester
Bennett, Michael V. L., Professor of Neuroscience, Director, Division of Cellular Neurobiology, Albert Einstein College of Medicine
Berg, Carl J., Jr., Research Associate, Harvard University
Bezanilla, Francisco, Professor, University of Chile, Chile
Bishop, Stephen H., Assistant Professor, Baylor College of Medicine
Blaustein, Mordecai P., Professor of Physiology and Biophysics, Washington University School of Medicine
Borgese, Thomas A., Associate Professor, Herbert H. Lehman College, The City University of New York
Borisy, Gary G., Professor of Molecular Biology and Zoology, University of Wisconsin
Boron, Walter F., Medical Scientist Training Program Fellow, Washington University School of Medicine
Bosler, Robert B., Principal Associate, Harvard Medical School
Brinley, F. J., Jr., Associate Professor of Physiology, The Johns Hopkins University
Brodwick, Malcolm S., Assistant Professor, University of Texas Medical Branch
Brown, Frank A., Jr., Morrison Professor of Biology, Northwestern University

Brown, Joel E., Professor of Physiology, State University of New York at Stony Brook
Burdick, Carolyn J., Associate Professor of Biology, Brooklyn College, The City University of New York
Burger, Max M., Chairman of the Biocenter, Biocenter of the University of Basel, Switzerland
Burgos, Mario H., Full Professor, Director, Instituto de Histologia y Embriologia, N. Cuyo University, Mendoza, Argentina
Calahan, Michael D., Postdoctoral Fellow, University of Pennsylvania
Cebra, John J., Professor of Biology, The Johns Hopkins University
Chang, Donald C., Assistant Professor, Baylor College of Medicine
Chappell, Richard L., Associate Professor, Hunter College, The City University of New York
Charlton, J. Sherwood, Research Associate, Marine Biological Laboratory
Claude, Philippa, Assistant Scientist, University of Wisconsin Primate Research Center
Cloud, Joseph G., Postdoctoral Fellow, The Johns Hopkins University
Cohen, Lawrence B., Associate Professor, Yale University School of Medicine
Cohen, Yehuda, Lecturer, Hebrew University, Jerusalem
Cole, Gerald A., Professor of Epidemiology, The Johns Hopkins University, School of Hygiene
Cole, Kenneth S., Research Biophysicist, National Institutes of Health
Cooperstein, Sherwin J., Professor of Anatomy, University of Connecticut
Cremer-Bartels, Gertrud, Priv. Dozent, University of Munster, West Germany
Crow, Terry J., Extramural Fellow, NINCDS, National Institutes of Health
Crowe, John H., Jr., Associate Professor of Zoology, University of California, Davis
DeFelice, Louis J., Associate Professor of Anatomy, Emory University
de Terra, Noel, Research Associate Professor, Hahnemann Medical College
DeWeer, Paul, Associate Professor of Physiology and Biophysics, Washington Medical School
DiPolo, Reinaldo, Associate Investigator, Instituto Venezolano de Investigaciones Cientificas, Venezuela
Dodge, Frederick A., Adjunct Professor, The Rockefeller University
Dowling, John E., Professor of Biology, Harvard University
DuBois, Arthur B., Professor of Epidemiology and Physiology, Yale University Medical School, and Director, John B. Pierce Foundation Laboratory
Dunham, Phillip B., Professor, Syracuse University
Eaton, Douglas C., Assistant Professor, University of Texas Medical Branch
Eberhard, Anatol, Associate Professor of Chemistry, Ithaca College
Edds, Kenneth T., Postdoctoral Associate, Marine Biological Laboratory
Ehrenstein, Gerald, Research Physicist, National Institutes of Health
Ellis, Richard J., Associate Professor, Bucknell University
Ellison, Rebecca P., Trainee, The Population Council, The Rockefeller University
Elvin, David W., Visiting Assistant Professor, University of Vermont
Epel, David, Professor of Biology, Scripps Institution of Oceanography
Fallon, John T., Research Fellow in Pathology, Harvard Medical School, Massachusetts General Hospital, Shriners Burns Institute
Farmanfarmaian, A., Professor of Physiology, Rutgers—The State University of New Jersey
Fischbach, Gerald, Associate Professor, Harvard Medical School
Fishman, George S., Professor of Operations Research and Systems Analysis, University of North Carolina at Chapel Hill
Fishman, Harvey M., Associate Professor of Physiology and Biophysics, University of Texas Medical Branch at Galveston
Fohlmeister, Jurgen, Lecturer, University of Minnesota
French, Robert J., Visiting Fellow, NINCDS, National Institutes of Health
Furshpan, Edwin J., Professor of Neurobiology, Harvard Medical School
Gainer, Harold, Head, Section of Functional Neurochemistry, NICHD, National Institutes of Health
Gascoyne, Peter, Research Student, University College of North Wales, United Kingdom
Gibson, Jane, Associate Professor of Biochemistry, Molecular and Cell Biology, Cornell University
Gilbert, Daniel L., Research Physiologist, NINCDS, National Institutes of Health
Gold, Kenneth, Research Ecologist, New York Zoological Society, New York Aquarium
Goldman, David E., Independent Investigator, Marine Biological Laboratory
Goldsmith, Timothy H., Professor of Biology, Yale University
Gordon, James, Associate Professor, Hunter College and The Rockefeller University

GREENBERG, MICHAEL J., Professor of Biological Sciences, Florida State University
GRINVALD, AMIRAM, Research Associate, Yale University School of Medicine
GROSCH, DANIEL S., Professor of Genetics, North Carolina State University, Raleigh
GROSSMAN, ALBERT, Professor, New York University Medical School
GROSSMAN, YORAM, Visiting Fellow, NINCDS, National Institutes of Health
GUERITAUD, JEAN P., Postdoctoral Fellow, Albert Einstein College of Medicine
GUTTMAN, RITA, Professor of Biology, Brooklyn College, The City University of New York
HALVORSON, HARLYN O., Director, Rosenstiel Basic Medical Sciences Research Center and Professor of Biology, Brandeis University
HAMKALO, BARBARA ANN, Assistant Professor of Biology, University of California, Irvine
HANNA, ROBERT B., Instructor of Neuroscience, Albert Einstein College of Medicine
HARDING, CLIFFORD V., Professor and Director of Research, Kresge Eye Institute, Wayne State University
HASCHEMEYER, AUDREY E. V., Professor of Biology and Biochemistry, Hunter College, The City University of New York
HELDMAN, ELIAHU, Staff Fellow, NINCDS, National Institutes of Health
HEREFORD, LYNNA M., Postdoctoral Associate, Brandeis University
HEUSER, JOHN, Assistant Professor, University of California, San Francisco
HEYER, CAROLYN K., Research Associate, Max-Planck Institute for Psychiatry, West Germany
HIGHSTEIN, STEPHEN M., Assistant Professor of Neurosciences, Albert Einstein College of Medicine
HILLE, BERTIL, Professor of Physiology and Biophysics, University of Washington School of Medicine
HOCHACHKA, P. W., Visiting Investigator, University of British Columbia, Canada
HOSKIN, FRANCIS C. G., Professor, Illinois Institute of Technology
HUANG, P. C., Associate Professor of Biochemistry, The Johns Hopkins University
HUMPHREYS, TOM, Associate Professor, University of Hawaii
ILAN, JOSEPH, Associate Professor of Anatomy, Case Western Reserve University, Medical School
INOUE, ISOA, Visiting Fellow, NIMH, National Institutes of Health
INOUE, SADAYUKI, Assistant Professor, McGill University, Canada
JENKINS, ROBERT A., Professor of Zoology, University of Wyoming
JOHNSON, RALPH G., Professor and Chairman, University of Chicago
JOSEPHSON, ROBERT K., Professor, University of California, Irvine
JUMBLATT, JAMES E., Assistant, Biocenter, University of Basel, Switzerland
KAMINER, BENJAMIN, Professor and Chairman, Boston University School of Medicine
KAMINO, KOHTARO, Research Staff, Yale University School of Medicine
KAPLAN, EHUD, Postdoctoral Fellow, The Rockefeller University
KEDES, LAURENCE H., Assistant Professor, Stanford University
KEETON, WILLIAM T., Professor and Chairman, Section of Neurobiology and Behavior, Cornell University
KENNEDY, MARY B., Postdoctoral Fellow, Harvard Medical School
KLEENE, KENNETH C., NIH Postdoctoral Fellow, University of Hawaii
KLEINSCHMIDT, JOCHEN, Postdoctoral Research Fellow, Laboratory of Sensory Physiology, Marine Biological Laboratory
KLINE, RICHARD P., NIH Postdoctoral Fellow, Harvard University
KOIDE, SAMUEL S., Associate Director, The Population Council, The Rockefeller University
KRAVITZ, EDWARD A., Professor of Neurobiology, Harvard Medical School
KRIEBEL, MAHLON E., Associate Professor, State University of New York, Upstate Medical Center
KRYSTAL, GERRY, Postdoctoral Fellow, University of California, Irvine
KUFFLER, STEPHEN W., John Franklin Enders University Professor, Harvard Medical School
KUHNS, WILLIAM J., Associate Professor of Pathology, New York University School of Medicine
LANDOWNE, DAVID, Associate Professor, University of Miami
LASH, JAMES W., Professor of Anatomy, University of Pennsylvania, School of Medicine
LASEK, RAYMOND J., Associate Professor, Case Western Reserve University
LASS, YORAM, Research Fellow, Harvard Medical School
LAUFER, HANS, Professor of Biology, University of Connecticut
LEADBETTER, E. R., Professor of Biology, Amherst College
LEE, JOHN J., Professor of Biology, City College, The City University of New York
LESTER, ROGER, Professor of Medicine, Chief, Gastroenterology, University of Pittsburgh School of Medicine

Levin, Jack, Associate Professor of Medicine, The Johns Hopkins University School of Medicine
Levinthal, Cyrus, Professor of Biological Sciences, Columbia University
Levy, Milton, Professor of Biochemistry, New York University School of Medicine
Lingrel, Jerry B., Professor of Biological Chemistry, University of Cincinnati, College of Medicine
Lipicky, Raymond J., Professor of Pharmacology and Medicine, Director of Clinical Pharmacy, University of Cincinnati
Liuzzi, Anthony, Associate Professor, University of Lowell
Llinas, R., Professor of Physiology and Biophysics, University of Iowa
Loewenstein, W. R., Professor and Chairman of the Department of Physiology and Biophysics, University of Miami, School of Medicine
Longo, Frank J., Associate Professor, University of Tennessee Center for the Health Sciences
Lorand, L. Professor of Biochemistry and Molecular Biology, Northwestern University
Lux, Hans Dieter, Head, Department of Neurophysiology, Max-Planck Institute for Psychiatry, West Germany
Marler, Peter, Professor of Ecology and Ethology, The Rockefeller University
Marzluff, William F., Jr., Assistant Professor, Florida State University
Mauzerall, David, Professor, The Rockefeller University
Mastroianni, Luigi, Jr., Wm. Goodell Professor and Chairman, Obstetrics and Gynecology, University of Pennsylvania
Mathews, Rita W., Senior Research Associate, Hunter College, The City University of New York
Matsumoto, Gen, Guest Worker, NIMH, National Institutes of Health
Matsumura, Fumio, Professor, University of Wisconsin
Metuzals, J., Professor in charge of the Electron Microscopy Unit, University of Ottawa, Canada
Metz, Charles B., Professor, University of Miami
Mied, Paul A., Postdoctoral Fellow, The Johns Hopkins University
Miller, R. S., Professor, Yale University
Moore, John W., Professor of Physiology, Duke University
Moore, Lee E., Associate Professor of Physiology, Case Western Reserve University
Mooseker, Mark S., Research Fellow, Harvard Medical School
Morgan, John R., Research Student, National Foundation for Cancer Research
Mote, Michael I., Associate Professor of Biology, Temple University
Mueller, Paul, Senior Staff Scientist and Professor of Biophysics, University of Pennsylvania, Eastern Pennsylvania Psychiatric Institute
Mullins, L. J., Professor of Biophysics and Chairman, University of Maryland School of Medicine
Murphy, Dennis J., Postdoctoral Student, University of Maryland
Musick, James R., Instructor, University of Utah
Nagel, Ronald L., Associate Professor, Albert Einstein College of Medicine
Narahashi, Toshio, Professor of Physiology and Pharmacology, Duke University Medical Center
Nicosia, Santo V., Assistant Professor of Obstetrics, Gynecology and Pathology, University of Pennsylvania School of Medicine
Nielsen, Jennifer B., Senior Research Associate, Hunter College, The City University of New York
Nishioka, David J., Postdoctoral Fellow, Scripps Institution of Oceanography
Noe, Bryan D., Assistant Professor of Anatomy, Emory University
O'Lague, Paul H., Assistant Professor of Biology, University of California, Los Angeles
Olmsted, Joanna B., Assistant Professor, University of Rochester
Osman, Richard W., Research Associate, University of Chicago
Oxford, Gerry S., Postdoctoral Research Fellow, University of North Carolina
Pant, Harish C., Senior Staff Fellow, NIMH, National Institutes of Health
Pappas, George D., Professor of Neurosciences and Anatomy, Albert Einstein College of Medicine
Pardy, Rosevelt L., Assistant Dean, School of Biological Sciences, University of California, Irvine
Patterson, John A., Research Associate, Hunter College, The City University of New York
Person, Philip, Medical Investigator, Veterans Administration Hospital, Brooklyn
Pethig, R., University Lecturer, University College of North Wales, United Kingdom
Pierce, Sidney K., Associate Professor, University of Maryland
Pohl, Herbert A., Professor of Physics, Oklahoma State University
Politoff, Alberto L., Assistant Professor, Boston University School of Medicine

Pollard, Harvey B., Senior Investigator and Medical Officer, National Institutes of Health
Pollard, Thomas D., Associate Professor, Harvard Medical School
Porter, Keith R., Chairman and Professor, Molecular, Cellular and Developmental Biology, University of Colorado, and Director, Marine Biological Laboratory
Potter, David D. Professor of Neurobiology, Harvard Medical School
Poussart, Denis, Associate Professor, Université Laval, Canada
Prendergast, Robert A., Associate Professor of Ophthalmology, Associate Professor of Pathology, The Johns Hopkins University
Prior, David J., Assistant Professor, University of Kentucky
Ramon, Fidel, Assistant Medical Research Professor, Duke University
Rebhun, Lionel I., Professor, University of Virginia
Reese, Thomas, Head, Section on Functional Neuroanatomy, National Institutes of Health
Reinisch, Carol L., Research Associate, Sidney Farber Cancer Center and Harvard Medical School
Requena, Associate Investigator, Instituto Venezolano de Investigaciones, Venezuela
Reynolds, George T., Professor of Physics, Princeton University
Rheuben, Mary B., Intergovernmental Exchange Biologist, nih, and Pennsylvania State University
Rice, Robert V., Professor and Head, Department of Biological Sciences, Mellon Institute of Carnegie-Mellon University
Ripps, Harris, Professor of Ophthalmology and Physiology, New York University School of Medicine
Ritchie, J. Murdoch, Professor and Director, Division of Biological Sciences, Yale University
Rosbash, Michael M., Assistant Professor of Biology, Brandeis University
Rose, Birgit, Assistant Professor, University of Miami, School of Medicine
Rosenbaum, Joel L., Associate Professor, Yale University
Rosenbluth, Jack, Professor of Physiology, New York University Medical Center
Roslansky, Priscilla F., Research Associate, Carnegie-Mellon University
Ross, William N., Postdoctoral Fellow, Harvard Medical School
Rubin, Lee L., Postdoctoral Fellow, Harvard Medical School
Ruderman, Joan V., Postdoctoral Fellow, Massachusetts Institute of Technology
Rushforth, Norman B., Professor and Chairman, Department of Biology, Case Western Reserve University
Russell, John M., Assistant Professor, University of Texas Medical Branch
Russell-Hunter, W. D., Professor of Zoology, Syracuse University
Salzberg, Brian Matthew, Assistant Professor of Physiology, University of Pennsylvania
Scarpa, Antonio, Associate Professor of Biochemistry and Biophysics, University of Pennsylvania
Schiff, Jerome A., Professor of Biology and Director of the Institute for Photobiology of Cells and Organelles, Brandeis University
Schopf, Thomas J. M., Associate Professor, University of Chicago
Schuel, Herbert, Associate Professor of Biochemistry, State University of New York, Downstate Medical Center
Schuetz, Allen W., Associate Professor, The Johns Hopkins University
Sears, James R., Assistant Professor of Biology, Southeastern Massachusetts University
Segal, Sheldon J., Vice President and Director, Biomedical Division, The Population Council, The Rockefeller University
Seyama, Issei, Postdoctoral Research Associate, Duke University Medical Center
Shapley, Robert, Assistant Professor, The Rockefeller University
Sherman, Irwin W., Professor of Zoology, University of California, Riverside
Shoukimas, Jonathan J., Postdoctoral Fellow, Laboratory of Biophysics, nincds, National Institutes of Health
Shrivastav, Brij Bhushan, Assistant Medical Research Professor, Duke University Medical Center
Siegel, Irwin M., Professor of Experimental Ophthalmology, New York University Medical Center
Smith, Frederick E., Professor of Resources and Ecology, Harvard University
Smith, Michael A. K., Research Associate, Hunter College, The City University of New York
Sobel, Matthew J., Associate Professor, Yale University
Spiegel, Evelyn, Research Associate, Dartmouth College

Spiegel, Melvin, Professor of Biology, Dartmouth College
Spira, Micha E., Visiting Assistant Professor, Albert Einstein College of Medicine
Spray, David C., Research Fellow, Albert Einstein College of Medicine
Starzak, Michael E., Assistant Professor, State University of New York at Binghamton
Steinacker, Antoinette, Postdoctoral Fellow, Albert Einstein College of Medicine
Stephens, Raymond E., Resident Investigator, Marine Biological Laboratory and Associate Professor of Biology, Brandeis University
Stetten, DeWitt, Jr., Deputy Director for Science, National Institutes of Health
Stetten, Marjorie R., Biochemist, NIAMDD, National Institutes of Health
Stevens, Charles F., Professor of Physiology, Yale University
Stickgold, Robert, Research Fellow, Harvard Medical School
Stokes, Darrell R., Assistant Professor, Emory University
Storey, Kenneth B., Assistant Professor of Zoology, Duke University
Stretton, Antony O. W., Associate Professor, University of Wisconsin
Strichartz, Gary R., Assistant Professor of Physiology and Biophysics, State University of New York at Stony Brook
Stuart, Ann E., Assistant Professor, Harvard Medical School
Stunkard, Horace W., Research Associate, American Museum of Natural History
Swarm, Richard L., Director of Experimental Pathology and Associate Director of Experimental Therapeutics, Hoffmann-La Roche. Inc Research Division
Szamier, Robert Bruce, Assistant Professor, Harvard Medical School
Szent-Györgyi, Albert, Principal Investigator, Marine Biological Laboratory
Szent-Györgyi, Andrew G., Chairman, Department of Biology, Brandeis University
Szent-Györgyi, Eva M., Research Associate, Brandeis University
Szuts, Ete Soltan, NIH Postdoctoral Fellow, Marine Biological Laboratory
Takashima, Shiro, Associate Professor, University of Pennsylvania
Tasaki, Ichiji, Chief, Laboratory of Neurobiology, NIMH, National Institutes of Health
Taylor, Robert E., Supervisor Research Physiologist, National Institutes of Health
Taylor, Roger B., Reader in Immunology, University of Bristol, United Kingdom
Tiffert, Teresa, Postdoctoral Fellow, The Johns Hopkins University
Trelstad, Robert L., Assistant Pathologist, Massachusetts General Hospital; Chief of Pathology, Shriners Burns Institute; Assistant Professor, Harvard Medical School
Trinkaus, J. P., Professor of Biology, Yale University
Troll, Walter, Professor of Environmental Medicine, New York University Medical School
Troxler, Robert F., Associate Professor of Biochemistry, Boston University School of Medicine
Vincent, Walter, Professor and Chairman, Department of Biological Sciences, University of Delaware
Wald, George, Professor of Biology, Harvard University
Walton, Alan, Lecturer, Open University, United Kingdom
Wang, Howard H., Assistant Professor of Biology, University of California, Santa Cruz
Warren, Leonard, Member, Wistar Institute, and Professor, University of Pennsylvania
Weber, Annemarie, Investigator, University of Pennsylvania
Weidner, Earl, Assistant Professor, Louisiana State University
Weissmann, Gerald, Professor of Medicine, Director, Division of Rheumatology, New York University School of Medicine
Wells, Jay B., Physiologist, National Institutes of Health
Whittaker, J. Richard, Associate Member, Wistar Institute
Wilson, Darcy B., Professor, University of Pennsylvania School of Medicine
Wittenberg, Jonathan B., Professor of Physiology, Albert Einstein College of Medicine
Wodlinger, Harold M., Graduate Student, Institute of Biomedical Engineering, University of Toronto, Canada
Wolf, Don P., Assistant Professor, University of Pennsylvania
Woodward, William R., Instructor, Harvard Medical School
Wu, Chau H., Medical Research Assistant Professor, Duke University Medical Center
Yeh, J. Z., Assistant Medical Research Professor, Duke University Medical Center
Yoshikami, Doju, Instructor, Harvard Medical School
Yoshioka, Tohru, Visiting Associate, NIMH, National Institutes of Health
Zigman, Seymour, Associate Professor of Ophthalmology and Biochemistry, University of Rochester School of Medicine and Dentistry

Zdunski, Dulcine, Postdoctoral Fellow, Stanford University
Zimmerberg, Joshua, Medical Scientist Trainee, Albert Einstein College of Medicine

Lillie Fellow, 1976

Paul, John, Director, Beatson Institute for Cancer Research, University of Glasgow, Scotland

Alexander Forbes Lecturer, 1976

Phillips, Charles, Oxford University, Oxford, United Kingdom

Rand Fellow, 1976

Lindenmann, Jean, Instructor, University of Zurich, Switzerland

Grass Fellows, 1976

Frazier, Donald T., Senior Fellow, Professor, University of Kentucky, School of Medicine
Abercrombie, Ronald F., Predoctoral Fellow, University of Maryland at Baltimore
Barton, Stuart B., Research Worker, University Laboratory of Physiology, Oxford, United Kingdom
Beiswanger, Christine M., Postdoctoral Fellow, University of Kentucky
Catapane, Edward J., Graduate Student, Fordham University
Finger, Thomas E., Postdoctoral Fellow, Washington University School of Medicine
Howe, Nathan R., Hopkins Marine Station, Stanford University
Johnston, Daniel, Assistant Professor, University of Minnesota
Kirsch, Glenn E., Grass Foundation Fellow, and Purdue University
Livingstone, Margaret S., Harvard Medical School
McCreery, Michael J., Postdoctoral Fellow, George Washington School of Medicine
Moody, William J., Jr., Graduate Student, Stanford University
Ornberg, Richard L., Graduate Student, Pennsylvania State University
Paton, John A., Postdoctoral Research Associate, Cornell University
Shecket, Gordon, Graduate Student, Case Western Reserve University

Macy Scholars, 1976

Adams, James A., Assistant Professor of Biology, Tennessee State University
Armstrong, Earlene, Assistant Professor, Howard University
Langford, George M., Assistant Professor of Biology, University of Massachusetts, Boston
Munavalli, S., Professor and Chairman, Chemistry Department, Livingstone College

Summer Research Scholarships, 1976
(Steps Toward Independence)

Bloodgood, Robert Alan, Postdoctoral Fellow, Yale University
Driedzic, William Richard, Postdoctoral Fellow, Pennsylvania State University
Harris, Gary C., Graduate Student, University of Massachusetts
Hobbs, Ann Snow, NIH Extramural Postdoctoral Fellow, NINCDS, National Institutes of Health
Howe, Craig W. S., Research Associate, Memorial Sloan-Kettering Cancer Center
Livengood, David R., Research Physiologist, Armed Forces Radiobiology Research Institute
Morris, Shirley M., Postdoctoral Research Fellow, Boston University
Pumplin, David W., Visiting Scientist, NINCDS, National Institutes of Health, and University of Puerto Rico

Research Assistants, 1976

Anderson, David J., Harvard University
Arias, Linda Nan, Mount Holyoke College
Babiarz Paula Susan, Syracuse University

Barkalow, Derek, Rutgers—The State University of New Jersey
Barrett, Martha B., University of Colorado
Beleckis, Robert A., University of Lowell
Belsky, Elizabeth, University of Pennsylvania
Bennett, Holly V. L., Albert Einstein College of Medicine
Bolanowski, Stanley J., Institute for Sensory Research, Syracuse University
Bryant, Bruce, Cornell University
Cartwright, Joiner T., University of Hawaii
Chalas, Eva, Albert Einstein College of Medicine
Cole, James J., Boston University Marine Program
Costa, Charles J., University of Maryland
Crisanti, Lynn, University of Pittsburgh
Csernansky, John, New York University
Deaton, Lewis, Florida State University
DeRosa, R., University of Toronto, Canada
Dominello, Geraldine, University of Connecticut Health Center
Donaldson, Patricia Lynn, University of California, Irvine
Donmoyer, Judith E., University of Wisconsin
Drabkin, Harold J., Wesleyan University
Duncan, Roger, University of Hawaii
Eberhard, Carolyn, Cornell University
Edgington, Duane R., Harvard University
Ellwood, Rosalind E., North Carolina State University
Epel, Debra L., Northwestern University
Fechheimer, Marcus, The Johns Hopkins University
Feldman, Barbara, Shriners Burns Institute
Feldman, Lance, University of Texas Medical Branch
Fitt, William K., Jr., University of California, Irvine
Fregien, Linda, University of Hawaii
Glad, Richard W., The Johns Hopkins School of Hygiene and Public Health
Greene, Sharon I., University of Virginia
Griffith, Linda M., Harvard University
Grob, Marianne, University of Basel, Switzerland
Hand, R. Taber, Pomona College
Hankins, Carol A., Simmons College
Harris, Andrew L., Albert Einstein College of Medicine
Hudson, Alan Paul, City University of New York
Hunter, Ann W., Emory University
Hurst, Terry W., University of Texas Medical Branch
Huse, William D., Albert Einstein College of Medicine
Israel, Alan, New York University School of Medicine
Jacobsen, Ronie, Dartmouth College
Jampel, Delia, Kresge Eye Institute
Jaslove, Stewart, Duke University Medical Center
Kaufmann, Karl, University of Chicago
Keller, Thomas C. S., III, University of Virginia
Keller, Laura R., University of Viriginia
Kiehart, Daniel P., University of Pennsylvania
Knowles, Cheryl A., Ithaca College
Kordik, Ellen R., Illinois Institute of Technology
Korman, Gail, Beth Israel Hospital
Kuhns, Fred, Harvard College
LaFratta, James M., Harvard Medical School
Lambertsen, Richard H., University of Pennsylvania
Laufer, Jessica, Brandeis University
Lifset, Reid J., Swarthmore College
Lotter, Shelley, Yale University
Lowenhaupt, Manuel T., Massachusetts Institute of Technology
Lowry, John T., Jr., Bucknell University

Madin, Katherine A. C., University of California, Davis
Markowitz, Leslie, Rutgers—The State University of New Jersey
Makaretz, Michael, Worcester Foundation Experimental Biology
Martin, Jill, University of Connecticut Health Center
Mathews, Sarah
Mastropaolo, Carmine, City College, The City University of New York
Miller, Celeste D.
Moore, Stephen T., Harvard University
Morales, Eleanor A., Osborn Laboratories of Marine Sciences, New York Aquarium, New York Zoological Society
Moshier, Jeffrey A., The Johns Hopkins University
Muratore, John F., Yale University
Narahashi, Keiko, Duke University
Olive, Cader W., Michigan State University
Osborn, Maria La Grange, University of Maryland
Parmentier, Carol A., Duke University
Paulson, Andrew M., Yale University
Perry, George, Scripps Institution of Oceanography
Perry, J. Gavin, Washington University Medical School
Persell, Roger A., Hunter College, The City University of New York
Reeves, Charlotte, Duke University
Reinhart, Thomas, Dartmouth College
Revel, David, University of California, San Diego
Richardson, Ann L., Louisiana State University
Rini, Frank J., Columbia University
Robertson, Lola E., American Museum of Natural History
Rogart, Richard B., Yale University School of Medicine
Romano, Frank A., III, Syracuse University
Rossi, Michael, Case Western Reserve University
Salem, Esther, Boston University
Saling, Patricia M., University of Pennsylvania
Samson, Douglas A., The Johns Hopkins School of Hygiene
Santiago, Eligio M., Washington University School of Medicine
Sass, Sherry L., Southeastern Massachusetts University
Schairer, John, Albert Einstein College of Medicine
Scherr, Les J., Albert Einstein College of Medicine
Scholtyssek, Birgit, Wilhelms-Universitat, Eye Hospital, West Germany
Scruggs, Virginia, University of Miami
She, Joseph, Scarborough College, University of Toronto, Canada
Siegel, Ruth E., Harvard Medical School
Smith, Glenn D., The Johns Hopkins University
Smith, Rick L., University of Hawaii
Smith, Tonicia M., Marine Biological Laboratory
Sobota, Mary J. University of Minnesota
Spindel, Lawrence M., Boston University School of Medicine
Stanchfield, James E., Dartmouth College
Stich, Thomas J., The Johns Hopkins University, School of Medicine
Sullivan, Brian, AMR Corporation
Taylor, Barbara A., Brooklyn College, The City University of New York
Theran, Ruthanne, St. Francis College
Tuna, Ishik C., The Johns Hopkins University
Tuttleman, Jan, Ithaca College
Vijverberg, Henk P. M., Duke University, and University of Utrecht, The Netherlands
Vincent, Walter S., Dartmouth College
Vonusa, Rosemarie
Walton, Kerry, University of Iowa
Watson, Patricia, The Johns Hopkins University
Weisman, Stuart, State University of New York at Stony Brook
Westerfield, Monte, Duke University Medical School

WHITE, ROY L., University of Illinois
WILSON, ANDREW H., JR., Worcester Foundation for Experimental Biology
WILTSE, WENDY, University of Massachusetts
WILNER, ANDREW N., Yale University
WRIGHT, MARY F., Wheelock College
YONEMOTO, WES, University of Hawaii
YULO, TERESA S., University of Rochester School of Medicine and Dentistry
ZAKEVICIUS, JANE M., New York University School of Medicine
ZIPKIN, PAUL H., Yale University
ZUKOWSKI, ANTHONY J., Marietta College

Library Readers, 1976

ADELBERG, EDWARD A., Professor of Human Genetics, Yale University
ALLEN, GARLAND E., Associate Professor of Biology and History of Science, Washington University
ANDERSEN, H. A., Research Fellow, Biological Institute of the Carlsberg Foundation
ARMSTRONG, CLARA FRANZINI, Associate Professor of Anatomy, University of Pennsylvania
BEALE, SAMUEL I., Assistant Professor, The Rockefeller University
BELL, EUGENE, Professor of Biology, Massachusetts Institute of Technology
BERG, HOWARD C., Professor of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder
BURR, ARTHUR H., Assistant Professor, Simon Fraser University, Canada
CARLSON, FRANCIS D., Professor of Biophysics, The Johns Hopkins University
CARRIERE, RITA, Assistant Professor, State University of New York, Downstate Medical Center
CHILD, FRANK M., Professor of Biology, Trinity College
CLARK, ARNOLD M., Professor of Biological Sciences, University of Delaware
CLIFFORD, SISTER ADELE, Professor of Biology, College of Mount St. Joseph
COLWIN, ARTHUR L., Adjunct Professor, University of Miami
COOPER, RICHARD A., Chief, Hematology-oncology, University of Pennsylvania
DEFENDI, VITTORIO, Professor and Chairman, Department of Pathology, New York University School of Medicine
DE TOLEDO, LEYLA, Associate Professor, Rush Medical College and University of Toronto
EDDS, LOUISE L., Research Associate, Children's Hospital Medical Center, Boston
EDER, HOWARD A., Professor of Medicine, Albert Einstein College of Medicine
EHRENSTEIN, GÜNTER VON, Director of the Department of Molecular Biology, Max-Planck Institute for Experimental Medicine, West Germany
ENGLANDER, S. W., Professor of Biochemistry and Biophysics, University of Pennsylvania
ETTIENNE, EARL M., Associate Professor of Physiology, University of Massachusetts Medical Center, Worcester
FELL, PAUL E., Associate Professor of Zoology, Chairman, Connecticut College
FESTOFF, BARRY W., Associate Professor, Chief, Neurology Service, Kansas University Medical Center, Kansas City VA Hospital
GABRIEL, MORDECAI L., Dean, School of Science, Brooklyn College, The City University of New York
GERMAN, JAMES L., III, Senior Investigator and Director, Laboratory of Human Genetics, The New York Blood Center, and Cornell University Medical College
GILLETTE, PETER, Assistant Professor of Medicine, State University of New York, Downstate Medical Center
GOLDSTEIN, MOISE H., JR., Professor, The Johns Hopkins University
GOUDSMIT, ESTHER M., Assistant Professor, Oakland University
GOULD, STEPHEN J., Professor of Geology, Harvard University
GREEN, JAMES W., Professor of Physiology, Rutgers—The State University of New Jersey
HANDLER, PHILIP, President, National Academy of Sciences
HILLMAN, PETER, Associate Professor of Biophysics, Hebrew University of Jerusalem, Israel
HINSCH, GERTRUDE W., Associate Professor, University of South Florida
HOPKINS, PENNY M., Assistant Professor, American Museum of Natural History, Ramapo College of New Jersey
HUNTER, R. DOUGLAS, Assistant Professor of Biological Sciences, Oakland University
ISENBERG, IRVIN, Professor of Biophysics, Oregon State University

Isselbacher, Kurt J., Mallinckrodt Professor of Medicine, Chief, Gastrointestinal Unit, Harvard Medical School and Massachusetts General Hospital

Kaiserman-Abramof, Ita R., Associate Professor of Anatomy, Case Western Reserve University, Medical School

Kaltenbach, Jane C., Professor of Biological Sciences, Mount Holyoke College

Karush, Fred, Professor of Microbiology, University of Pennsylvania School of Medicine

Keosian, John, Professor Emeritus, Rutgers—The State University of New Jersey

Kosower, Edward M., Professor of Chemistry, Tel-Aviv University, Israel

Krupa, Paul L., Associate Professor of Biology, City College, The City University of New York

Laderman, Aimlee D., Graduate Student, Doctoral Candidate, State University of New York at Binghamton

Laster, Leonard, Vice-President for Academic and Clinical Affairs, Dean, College of Medicine, State University of New York, Downstate Medical Center

Leighton, Joseph, Professor and Chairman, Department of Pathology, Medical College of Pennsylvania

Levitan, Herbert, Associate Professor of Biology, University of Maryland

Lutzner, Marvin A., Chief, Dermatology Branch, NCI, National Institutes of Health

Marsland, Douglas, Research Professor (Retired), New York University

Mizell, Merle, Professor of Biology, Tulane University

Morrell, Frank, Professor of Neurology, Rush Medical College

Munavalli, S., Professor and Chairman, Chemistry Department, Livingstone College

Palmer, John D., Professor and Chairman, University of Massachusetts

Pearlman, Alan L., Associate Professor of Physiology and Neurology, Washington University School of Medicine

Plocke, Donald J., S. J., Chairman, Department of Biology, Boston College

Potter, H. David, Associate Professor, Neural Sciences and Zoology, Indiana University

Prusch, Robert D., Assistant Professor, Biomedical Sciences, Brown University

Przybylski, Ronald J., Associate Professor, Case Western Reserve University

Reiner, John M., Professor of Biochemistry, Research Professor of Pathology, Albany Medical College of Union University

Ricklefs, Robert E., Associate Professor of Biology, University of Pennsylvania

Rosenberg, Evelyn Kivy, Professor of Biology Department, Jersey City State College

Rosenkranz, Herbert S., Professor and Chiarman, Department of Microbiology, Adjunct Professor of Pediatric Research, New York Medical College, Columbia University

Rowland, Lewis P., Professor and Chairman, Department of Neurology, Columbia University

Rubinow, Sol I., Professor of Biomathematics, Cornell University Medical College

Saunders, John W., Jr., Professor of Biological Sciences, State University of New York at Albany

Schindler, Gerda, Psychology Intern, Postgraduate Center of Mental Health

Schlesinger, R. Walter, Professor and Chairman, Department of Microbiology, Rutgers University Medical School

Scott, George T., Professor of Biology, Oberlin College

Shedlovski, Theodore, Professor Emeritus, The Rockefeller University

Shelanski, Michael L., Associate Professor of Neuropathology, Harvard Medical School

Shemin, David, Professor and Chairman, Department of Biochemistry and Molecular Biology, Northwestern University

Shepro, David, Professor, Boston University

Sly, William S., Professor of Pediatics and Medicine, Director, Division of Medical Genetics, Washington University School of Medicine

Sonnenblick, B. P., Professor of Zoology and Physiology, Rutgers—The State University of New Jersey

Speck, William T., Assistant Professor of Pediatrics and Microbiology, Columbia University, College of Physicians and Surgeons

Stone, William, Jr., Director, National Institute of Scientific Research

Sussman, Maurice, Chairman, Department of Life Sciences, University of Pittsburgh

Tilney, Lewis, Associate Professor of Biology, University of Pennsylvania

Tweedell, Kenyon S., Professor, University of Notre Dame

Van Holde, K. E., Professor of Biophysics, Oregon State University

Wainio, Walter, Professor and Chairman, Biochemistry, Rutgers—The State University of New Jersey

Waite, Thomas D., Assistant Professor of Civil Engineering, Head, Environmental Engineering Program, University of Miami
Waksman, Byron H., Professor of Pathology, Yale University
Webb, H. Marguerite, Professor of Biological Sciences, Goucher College
Weiss, Leon, Professor and Chairman, Department of Animal Biology, University of Pennsylvania
Wheeler, George E., Professor of Biology, Brooklyn College, The City University of New York
Whittingham, David Gordon, Scientific Staff Member, Medical Research Council—Mammalian Development Unit, United Kingdom
Wilson, Thomas H., Professor of Physiology, Harvard Medical School
Wittenberg, Beatrice A., Associate Professor of Physiology, Albert Einstein College of Medicine
Yntema, Chester L., Professor Emeritus, State University of New York
Zacks, Sumner I., Neuropathologist, Pennsylvania Hospital and Professor of Pathology, University of Pennsylvania School of Medicine
Zuckerkandl, Emile, Visiting Professor, University of Delaware, and Directeur de Recherche, CNRS, France

Students, 1976

All students listed completed the formal course programs. Asterisk indicates completing post-course research programs.

Summer Programs 1976

ECOLOGY

Andrews, Daniel
Boone, Richard D.
Brown, Aaron D.
Cooper, Rosalind (Sally) B.
de Mello, Richard P.
Fagerness, Vicki L.
Gilinsky, Ellen
Maurer, David R.
McBride, Margaret M.
McCoy, George L.
McNamara, Scott J.
Miller, Lynne M.
Ryan, Patrick F.
Scholl, James E.
Schwartzkopf, Steven H.
Semetko, Kathryn Ann
Sullivan, Patricia E.
Wyman, Charles D.

EMBRYOLOGY

Baran, Madelyn M.
Bloom, Kerry S.
Braun, Barbara A.
Bremner, Theodore A.
Cheney, Clarissa M.
Flecha, Miguel
Fregien, Nevis L.
French, Cynthia K.
Fyrberg, Eric A.
Golden, Lynn
Griffiths, Jeffrey K.
Hall, Terrence J.
Marchionni, Mark
Marco, Roberto
Merrifield, Peter A.
Nagle, Barbara W.
Parsons, Bruce
Phillips, Carey R.
*Pogel, Burton
*Rosenspire, Allen
States, David J.
Steele, Robert E.
Vaslet, Charles A.
Vito, Christine C.

EXPERIMENTAL INVERTEBRATE ZOOLOGY

*Arceneaux, Mila L.
*Augustine, George J., Jr.
*Battey, James F.
*Becker, Anthony J., Jr.
Bergstrom, Peter W.
Bertoldi, Roger V.
*Biela, Christina M.
Burford, David R., Jr.
*Burke, Elaine M.
*Chaisson, Richard E.
*Colón, Rita D.
*Cook, George A.
Cooper, Valerie F.
*Dickson, Kathryn A.
*Douglas, Ruth A.
Earl, Christopher D.

Fetterer, Raymond H., Jr.
*Greene, Sharon I.
Grega, Debra S.
Lansman, Jeffry B.
*Leitschuh, Mary K.
*Loest, Robert A.
Maxwell, Catherine S.
Miner, Martin M.
*Orida, Norman K.
Otto, James
Parsons, Pauline G.
Rome, Lawrence C.
*Schneiderman, Anne M.
*Serunian, Leslie A.
Sweeney, Thomas E.
*Vogt, Richard G.
Williams, Marta G.
Williamson, Craig E.
Yamin, Michael A.

EXPERIMENTAL MARINE BOTANY

*Collins, Yvonne
*Cosner, Jill
*Gale, James M.
Guarino, Linda A.
*Herz, Jeffrey M.
Howard, Diana
*Lobban, Christopher S.
Meeson, Blanche W.
*Mishkind, Michael L.
Moshkovski, Dror
*Pocratsky, Linda
*Shure, Richard L.
*Staiano, Lisa F.
Swanberg, Neil R.
Wolfe, James M.
Zullo, James F.

NEUROBIOLOGY

Damassa, David A.
Davis, Thomas L.
Fine, Alan
Fritz, Lawrence C.
Gitschier, Jane M.
Hawkins, Morris, Jr.
Johnston, Michael F.
Pardo, Jose V.
Schehr, Robert S.
Shotton, David M.
Siegel, Sharon B.
Weill, Cheryl L.

PHYSIOLOGY

* Aft, Rebecca L.
*Begg, David A.
*Boardman, Mark A.
Cawley, Michael F.
Chao, Nelson Jen An
*Cooper, David L.
*Dangott, Lawrence J.
Eckhardt, Laurie A.
Ellenbogen, Kenneth A.
*Fowler, Walter E.
*Gehrke, Lee
*Giantini, Michael
*Goetz, George H.
*Hansen, Dale A.
*Hatch, Christopher L.
*Haynes, John K.
*Herman, Ira M.
Hsu, Ellen
Jackson, Susan
Katzel, Leslie I.
*Kaufman, Harvey W.
*Kielian, Margaret C.
*Lisak, James C.
*Moore, Patrick L.
Mordan, Lawrence J.
Patterson, Thomas A.
*Pratt, Melanie M.
*Price, Maureen G.
Redner, Robert L.
*Roberson, Marie M.
Ronnett, Gabriele V.
*Ryerse, Jan S.
Sundborg, Michael A.
*Triplett, Barbara A.
Underwood, Eileen M.
Vafopolou-Mandalos, Xanthe
*Wang, Nancy S.
Wax, Amy L.
*Wilson, Douglas R.
*Wysocki, Lawrence J.
*Zerial, Aurelio

January Programs 1976

BEHAVIOR

Ackerman, William L.
Blatt, Gene J.
Bowles, Rebecca R.
Collins, Linda S.

Colomb, Etienne
Coon, Thomas G.
Dowd, Christina
Dyer, Betsey D.
Kolba, Clifford A.
Maciolek, Nancy J.
Miller, Robert E.
Nicholson, Deborah S.
Pitler, Timothy D.
Prince, Cameron E.
Seaborn, Charles R.
Stranger, Carolyn M.
Zimmer, Richard K.

DEVELOPMENTAL BIOLOGY

Arpke, Beth G.
Baird, Wilbur L.
Barrilleaux, Christopher N.
Binder, Robert L.
Birchard, Geoffrey F.
Enenstein, Judy
Ferl, Robert J.
Herbert, Pamela
Laureno, Kenneth
Lipshultz, Steven E.
Mazocco, Michael D.
Middaugh, Robert E.
Moreau, Pierre
Morelli, Thomas A.
Oschsner, Jessica C.
Ptak, John A.
Raffio, Nancy
Rains, Ross
Reinhart, Thomas E.
Reiss, Paul M.
Richards, Henry M.
Roberts, Christopher W.
Ross, Marian S.
Rosenberg, Michael P.
Rothman, Mark A.
Schumacher, Christine M.
Shore, Scott K.
Smith, Nelson W.
Solomon, Howard M.
Strassman, Jeffrey C.
Van Dover, Cindy L.
Wadsworth, Patricia

ECOLOGY

Ashley, Peter J.
Bablanian, Gayne M.
Brokaw, Richard S., Jr.
Emslie, Margaret W.
Frede, Sascha
Houk, Kathleen A.
Hurd, Richard M.
Jepson, Karen A.
Kawano, Linda S.
Kessler, Steven J.
Lyerla, JoAnn D.
Miller, Virginia M.
Moss, David E.
Motten, Alexander F.
Newell, Sandra J.
Patton, Ernest G.
Pierce, Barry A.
Pressley, Thomas A.
Ready, Neal E.
Richmond, Robert H.
Schaffner, Linda C.
Sims, Naomi
Smith, Francie L.
Snow, Allison A.
Spencer, Craig N.
Sussman, Richard
Vaughan, Joseph K.
Werner, Susan M.
Zowada, Stephanie

NEUROBIOLOGY

Berman, Howard M.
Bertoldi, Roger
Buskirk, Daniel R.
Cohen, Bruce N.
Eisen, Judith S.
Espinosa E., Jose I.
French, Kathleen A.
Greene, Sharon I.
Gerking, Andrew A.
Gregg, Robert
Hayward, Russell A.
Johnson, Harold E., Jr.,
Kaminer, Brian
Kuhls, Thomas L.
Lansman, Jeffrey B.
Levy Edward J.
Mitz, Andrew R.
Paley, David A.
Pappas, Georgia
Pascoe, Natalie G.
Reiser, Peter J.
Sajovic, Peter
Spool, Peter R.
Stenzler, Daniel

YEAR-IN-SCIENCE

Fall 1975

Richard D. Boone
Linda S. Collins
Bruce F. Leon
Kathryn K. Matthew
Cindy L. Van Dover

Spring 1976

Timothy B. Brown
Linda S. Collins
Richard M. Hurd
Barry A. Pierce
Cindy L. Van Dover

3. Scholarships, 1976

Bio Club Scholarship:	Dror Moshkovski	James F. Zullo
Gary H. Calkins Scholarship:	Elaine M. Burke	
Lucretia Crocker Scholarship:	James M. Wolfe	
Macy Students:	Theodore Bremner Yvonne Collins Rita Colón	Morris Hawkins John Haynes

4. Training Programs

Research Program in Microbial Ecology

I. Instructors

Yehuda Cohen, The Hebrew University, Jerusalem
Jane A. Gibson, Cornell University
Holger W. Jannasch, Woods Hole Oceanographic Institution
Edward R. Leadbetter, Amherst College

II. Consultants

Harlyn O. Halvorson, Brandeis University
J. Woodland Hastings, Harvard University
Roger Y. Stanier, Institut Pasteur, Paris
Edward O. Wilson, Harvard University

III. Staff Associates

Richard P. Blakemore, Woods Hole Oceanographic Institution
James G. Ferry, University of Georgia
Kenneth H. Nealson, Scripps Institution of Oceanography
Jeanne S. Poindexter, Marymount College
Craig D. Taylor, Woods Hole Oceanographic Institution

IV. Special Lecturers

Arnold L. Demain, Massachusetts Institute of Technology
Henry Ehrlich, Rensselear Polytechnic Institute
Joel C. Goldman, Woods Hole Oceanographic Institution
J. Woodland Hastings, Harvard University
Galen E. Jones, University of New Hampshire
John W. Kanwisher, Woods Hole Oceanographic Institution
Norman Lazaroff, State University of New York at Binghamton
Beverly J. Pierson, University of Puget Sound, Tacoma

Daniel Pope, Rensselear Polytechnic Institute
John H. Ryther, Woods Hole Oceanographic Institution
Robert D. Simon, University of Rochester
Helen S. Vishniac, University of Rochester
John B. Waterbury, Woods Hole Oceanographic Institution
Meyer J. Wolin, New York Department of Public Health, Albany

V. Trainees

Lisa Anderson
*Barbara A. Hulick
*Steven J. LaCroix
*John A. Leigh
Beverly J. K. Pierson
*James P. Robeson
*Mohammed A. Rouf
Edward G. Ruby
*Richard Sussman

VI. Lectures

H. W. Jannasch	Introduction to microbial ecology I and II
H. W. Jannasch	Continuous culture in microbial ecology
H. W. Jannasch	Theory and practice of the chemostat
H. W. Jannasch	Experiments in deep sea microbiology
E. R. Leadbetter	The aerobic way of life
E. R. Leadbetter	Microbial attack of hydrocarbons
E. R. Leadbetter	Enrichment approaches to ecological analysis
J. A. Gibson	Photosynthetic bacteria I
J. A. Gibson	Nutrient uptake and its measurement
Y. Cohen	Photosynthetic bacteria II
Y. Cohen	The Solar Lake
Y. Cohen	The Solar Lake algal mats
J. G. Ferry	The microbial synthesis of acetate from CO_2
J. G. Ferry	Anaerobic microbial food chains
C. D. Taylor	The biology of methane-producing bacteria
C. D. Taylor	Interaction of sulfate reduction and methane production
R. P. Blakemore	Effects of PCBs on marine bacteria
R. P. Blakemore	Bacterial magnetotaxis
J. S. Poindexter	The prosthecate bacteria: where answers why
J. S. Poindexter	The Caulobacter stalk and its development
J. S. Poindexter	The viruses of the Caulobacter group
M. J. Wolin	The anaerobic way of life
M. J. Wolin	Species interaction in anaerobic systems
H. S. Vishniac	Marine fungi
H. S. Vishniac	Yeasts from the Antarctic
K. H. Nealson	Luminescent bacteria and their symbiosis with luminous fish
A. L. Demain	Production and possible function of extracellular antimicrobial metabolites
H. Ehrlich	The bacterial role in the genesis of manganese nodules
J. C. Goldman	Continuous culture of photosynthetic organisms
J. W. Hastings	The biology of bacterial luminescence
G. E. Jones	The microbial sulfur cycle in Oyster Pond
J. W. Kanwisher	Sense and nonsense in modern ecology
N. Lazaroff	Photocontrol of morphogenesis in blue-greens
B. J. Pierson	Chloroflexux, a gliding photosynthetic bacterium
D. Pope	The effect of hydrostatic pressure on microbial systems
J. H. Ryther	Aquaculture
R. D. Simon	Cyanophycin granules
J. B. Waterbury	Biology of blue-greens

5. Tabular View of Attendance, 1972–1976

	1972	*1973*	*1974*	*1975*	*1976*
Investigators—Total	561	523	508	511	535
Independent	328	312	302	301	312
Library Readers	76	86	75	81	93
Research Assistants	157	125	131	129	130
Students—Total	119	123	146	200	237
Summer courses					
Experimental Invertebrate Zoology	38	32	30	34	36
Embryology	19	20	21	24	24
Physiology	31	41	40	33	41
Experimental Marine Botany	14	15	11	14	16
Ecology	17	15	14	18	18
Neurobiology	8	12	12	12	12
January courses					
Developmental Biology			30	20	32
Behavior				17	17
Biosphere				17	
Ecology					29
Neurobiology				23	24
Trainees—Total	38	38	41	31	9
Total Attendance	726	696	707	754	793
Less persons represented in two categories	1	0	0	0	1
	725	696	707	754	792
Institutions Represented—Total	210	239	222	237	234
Foreign Institutions Represented	25	40	31	26	33

6. Institutions Represented, 1976

Alabama, University of
Albert Einstein College of Medicine
AMR Corporation
American Museum of Natural History
Amherst College
Arizona, University of
Armed Forces Radiobiology Research Institute
Baruch College
Baylor College
Beth Israel Hospital
Bloomsburg State College
Boston College
Boston University
Boston University School of Medicine
Brandeis University
Brooklyn College, The City University of New York
Brown University
Bucknell University
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, San Francisco
California, University of, Santa Barbara
California, University of, Santa Cruz
Cape Cod Community College
Carnegie-Mellon University
Case Western Reserve University
Case Western Reserve University, Medical School
Children's Hospital Medical Center, Boston
Chicago, University of
Cincinnati, University of
Cincinnati, University of, College of Medicine
City College, The City University of New York
Clark University
Colby College
College of Mount St. Joseph on the Ohio
College of St. Rose
Colorado, University of
Colorado, University of, Medical Center
Colorado College

Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Connecticut College
Cornell University
Cornell University Medical College
Dakota Wesleyan University
Dartmouth College
Delaware, University of
Denison University
Drew University
Duke University
Duke University Medical Center
Eastern Pennsylvania Psychiatric Institute
Eastman Kodak Co.
Eisenhower College
Emory University
Fairleigh Dickinson University
Fitchburg State College
Florida State University
Fordham University
George Mason University
George Washington School of Medicine
Georgetown University
Georgia, University of
Goucher College
Hahnemann Medical College
Hampshire College
Harvard Medical School
Harvard University
Hawaii, University of
Herbert Lehman College, The City University of New York
Hiram College
Hoffmann-LaRoche, Inc., Research Division
Hollins College
Howard University
Hunter College, The City University of New York
Illinois, University of
Illinois Institute of Technology
Indiana University
Institute for Sensory Research
Iowa, University of
Ithaca College
John B. Pierce Foundation Laboratory
Johns Hopkins University, The
Johns Hopkins University, The, School of Hygiene
Kansas City VA Hospital
Kansas State University
Kansas University Medical Center
Kent State University
Kentucky, University of
Kresge Eye Institute, Detroit
Laboratory of Sensory Physiology
Livingstone College
Louisiana State University
Lowell, University of
Luther College
Maine, University of, Orono
Marine Research, Inc.
Marietta College
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of, Amherst
Massachusetts, University of, Boston
Massachusetts, University of, Medical Center, Worcester
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Pennsylvania
Medical University of South Carolina
Mellon Institute of the Carnegie-Mellon University
Memorial Sloan-Kettering Cancer Center
Miami, University of
Miami, University of, School of Marine and Atmospheric Science
Miami, University of, School of Medicine
Miami University
Michigan, University of
Michigan State University
Minnesota, University of
Moravian College
Mount Holyoke College
Nasson College
National Academy of Sciences
National Foundation for Cancer Research
National Institute of Arthritis, Metabolism and Digestive Diseases
National Institute of Child Health and Development
National Institute of Mental Health
National Institute of Neurological Communicative Diseases and Stroke
National Institute of Scientific Research
National Institutes of Health
National Marine Fisheries Service
New College
New York Aquarium
New York Blood Center, The
New York Medical College
New York University
New York University Medical Center
New York University School of Medicine
New York Zoological Society
Nicholls State University
North Carolina, University of, at Chapel Hill
North Carolina State University, Raleigh
Northeast Fisheries Center
Northeastern University
Northwestern University
Notre Dame, University of
Oakland University
Oberlin College

Oklahoma, University of
Oklahoma State University
Oregon, University of
Oregon State University
Pacific, University of the
Pennsylvania, University of
Pennsylvania, Hospital of the University of
Pennsylvania, University of, School of Medicine
Pennsylvania State University
Pittsburgh, University of
Pittsburgh, University of, School of Medicine
Pomona College
Population Council, The
Postgraduate Center of Mental Health
Princeton University
Puget Sound, University of
Purdue University
Ramapo College of New Jersey
Reed College
Rhode Island, University of
Rochester, University of
Rochester, University of, School of Medicine and Dentistry
Rockefeller University, The
Rush Medical College
Russell Sage College
Rutgers—The State University of New Jersey
Rutgers University Medical School
St. Francis College
St. Lawrence University
Scripps Institution of Oceanography
Shriners Burns Institute
Sidney Farber Cancer Center
Simmons College
South Carolina, University of
South Florida, University of
Southeastern Massachusetts University
Stanford University
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Binghamton
State University of New York at Buffalo
State University of New York at Plattsburgh
State University of New York at Stony Brook
Swarthmore College
Syracuse University
Syracuse University, Laboratory of Sensory Communication
Temple University
Tennessee, University of
Tennessee Center for Health Services, University of
Tennessee State University
Texas, University of
Texas, University of, Medical Branch
Texas Christian University
Toledo, University of
Trinity College, Hartford
Tulane University
Union University, Albany Medical College of
Upsala College
Utah, University of
Utah State University
Vassar College
Vermont, University of
Veteran's Administration Hospital, Brooklyn
Virginia, University of
Washington, University of
Washington State University
Washington University, St. Louis
Washington University, School of Medicine
Wayne State University
Wesleyan University
West Virginia State College
Wisconsin, University of
Wistar Institute
Wofford College
Woods Hole Oceanographic Institution
Worcester Foundation Experimental Biology
Wyoming, University of
Yale University
Yale University School of Medicine

FOREIGN INSTITUTIONS REPRESENTED, 1976

Autonoma, University of, Spain
Basel, The University of, Switzerland
Bristol, University of, United Kingdom
British Columbia, University of, Canada
Carlsberg Foundation, Biological Institute of the, Denmark
Central Baptist Seminary, Canada
Centre National de la Recherche Scientifique, France
Chile, University of, Chile
Dalhousie University, Canada
Glasgow, University of, Scotland
Hebrew University, The, Israel
Institute for Marine Environmental Research, United Kingdom
Instituto Venezolano de Investigaciones, Venezuela
Lausanne, University of, Switzerland
Macdonald College of McGill University, Canada
Max-Planck Institute for Experimental Medicine, West Germany
Max-Planck Institute for Psychiatry, West Germany
McGill University, Canada
Medical Research Council—Mammalian Development Unit, United Kingdom
Mexico National University, Mexico
Munster, University of, West Germany
N. Cuyo University, Mendoza, Argentina

North Wales, University College of, United Kingdom
Open University, United Kingdom
Ottawa, University of, Canada
Puerto Rico, University of, at Rio Piedras
Simon Fraser University, Canada
Tel-Aviv University, Israel
Toronto, University of, Canada
Université Laval, Canada
University Laboratory of Physiology, United Kingdom
Utrecht, University of, The Netherlands
Western Ontario, University of, Canada
Wilhelms-Universitat, Eye Hospital, West Germany
Zurich, University of, Switzerland

7. Friday Evening Lectures, 1976

June 25

Arnold J. Levine .... Mechanisms of tumorigenesis of the small DNA tumor viruses
Princeton University

July 2

Howard C. Berg .... How bacteria move about
University of Colorado

July 8

Charles Phillips .... Electrical stimulation of nerve centers I
Oxford University, England;
Alexander Forbes Lecturer, MBL

July 9

Charles Phillips .... Electrical stimulation of nerve centers II
Oxford University, England;
Alexander Forbes Lecturer, MBL

July 16

Mary Lou Pardue .... Structure and function in *Drosophila* chromosomes
Massachusetts Institute of Technology

July 23

Frank Ruddle .... Cell genetic studies in man and mouse
Yale University

July 30

Frederik B. Bang .... Invertebrate pathology and human disease
The Johns Hopkins University

August 5

John Paul .... Molecular biology of erythropoiesis
The Beatson Institute for Cancer Research, University of Glasgow;
Lillie Fellow, MBL

August 13

Jean Lindenmann .... Inborn resistance to influenza virus—a failure story
University of Zürich; Rand Fellow, MBL

August 20

Peter Satir .... Control of microtubule sliding in cilia
University of California, Berkeley

August 27

Richard Eakin .... "Glimpses of my life's work" by "Hans Spemann" (an impersonation)
University of California, Berkeley

8. Members of the Corporation, 1976

Including Action of 1976 Annual Meeting

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VI. REPORT OF THE LIBRARIAN

At the beginning of the year the Library Committee members were asked to cut $20,000 from the annual subscription budget. A complete list of all journal titles, both MBL and WHOI, was circulated to the Committee before the summer meetings. Fortunately for the collection, all members resisted strenuously and, as a consequence, not one subscription was cancelled. However, in anticipation of such a cut, very few new books were ordered by the Laboratory, but the book collection did not suffer. The summer book exhibitors were very generous with their gifts of new books (in lieu of a fee for space) and the Woods Hole Oceanographic Institution spent $17,211 for library books; $3,200 of that amount for sorely needed reference books.

Our library places an even increasing burden on Laboratory funds, and this year the Library Committee was asked to draft a proposal that would involve both the MBL and WHOI in the responsibility of maintaining the collection at its present level of excellence. Many meetings were held and a final proposal was presented at the Annual Meeting in August. Lively debate ensued, and the Corporation Meeting was re-convened three times before final adjournment. At issue was the Library becoming a separate Corporation supported by both MBL and WHOI and governed by a Board of Trustees drawn from members of both institutions. By the end of the year no definite decision had been reached, but further proposals have been submitted and discussion is continuing.

The most valuable volumes in the collection are now protected and housed in a larger Rare Books Room on the third floor—the old xerox room. This move was made possible by a very generous gift from an anonymous donor. The present Rare Books Room in the library offices had shelf space for only 500 volumes. The new area can shelve 3,000 volumes. A security system has been installed and the oldest and most valuable journal volumes are now in this area. Dr. Garland Allen, an expert in the History of Science, donated much of his time helping us determine which volumes should be moved to this "secure" area.

Space is still one of our major problems. Room 306, which was renovated to aid with the increased volume of book purchases by the Oceanographic, is just about full, and some new journal sets in the Marine Policy section have had to be shelved there. Three new journal sets (large gifts) are shelved on the counters in the stacks awaiting major moves in the journal collection.

Interlibrary loan requests increased again this year: approximately 7,000 requests for articles were filled in 1976. Our copy department processed 660,000 sheets of xerox during the year, and we now have two staff members handling this operation on a year-round basis. Two operators were added during the peak summer months. Total number of volumes in the collection at the end of 1976 is 161,864.

VII. REPORT OF THE TREASURER

The Marine Biological Laboratory had an excess of revenues over expenditures (excluding depreciation) of $106,197 in 1976 as compared to a deficit of $318,943 in 1975. The favorable difference of $425,140 reflects in large measure an increase in overhead related to research space of $308,248. The staff deserves great credit for their convincing presentation of the effect of the Laboratory's seasonal pattern of activity upon its costs of operation, which resulted in an increase in the allowed overhead rate from $16.23 to $31.29, a 93% increase. The change is retroactive to January 1, 1975. Accordingly, $110,631 of revenue recognized in 1976 is in fact related to 1975.

It has been pointed out in the past that the control of expenditures at the Laboratory has been effective, particularly taking into account inflation and its effect upon almost all elements of costs. However, prior to 1976, income had not kept pace with expenses resulting in annual cash deficits beginning in 1969. It is therefore gratifying to see the progress made in 1976 to correct this imbalance.

In addition to the increase in overhead rates, revenues also reflect first-time support for the Library in the amount of $10,000 each from the U. S. Fisheries and U. S. Geological Survey. The Woods Hole Oceanographic Institution increased its support from $20,000 to $30,000.

Income from Research Services (including the Supply Department) increased by $37,675 from $181,200 to $218,875.

Unrestricted gifts increased by $45,501 from $50,684 to $96,185 due in large measure to increased support from foundations and corporations.

Investment income for the year (on both unrestricted and restricted funds) amounted to $302,322 as compared to $261,540 in 1975, an increase of $40,782 or 16%. This represents a cash yield of 5.8% on the cost of the underlying investments. This yield reflects the percentage of the Laboratory's portfolio invested in common stocks. In addition to investment income, there were realized gains on the sales of investments of $175,732 which go to increase the Laboratory's endowment funds.

Grants and gifts of $1,549,931 were received in 1976, compared to $1,805,397 in 1975. Of the 1976 amount, $1,453,746 were designated to be used for specific programs. At year end 1976, there remained $1,279,573 in pledges for specific programs of the Laboratory ($887,109 at December 31, 1975). These amounts are scheduled to be received in the years 1977 to 1980 inclusive.

A year ago, the role of the MBL Associates in making possible the very attractive landscaping of the Quadrangle was recognized. In 1976 the Associates provided further funds, the income from which will go to maintain the area.

It should be noted here that the comparison of revenues and expenses for 1976 and 1975 is distorted by the effect of the change in the operation of the dining hall from the meal ticket system used in 1975 (under which the Laboratory collected the price of meal tickets and reimbursed the dining hall operator) to the "a la carte" system in 1976, with users making payment for meals directly to the operator. This resulted in a $111,790 decrease in revenues, partially offset by a $97,753 decrease in dining hall operating expenses.

Adjusted for these changes, total operating expenses increased by $52,633 from $2,937,494 to $2,990,127. This was in turn the result of a decrease in unrestricted operating expenses of $170,705 and an increase in expenditures for designated purposes (*i.e.*, restricted) of $223,338.

The decrease in unrestricted expenses reflects a $45,918 decline in repairs and remodeling and a $36,262 difference resulting from the write-off in 1975 of old issues of *The Biological Bulletin*. As can be seen, apart from these items, unrestricted expenses were still below the 1975 level with, of course, increases and decreases in specific categories of expense.

The increase in expenditures for designated purposes from $952,464 in 1975 to $1,175,802 in 1976, an increase of $223,338 or 23%, reflects in large part an increased level of activity in The Ecosystems Center, together with the addition of several new research grants or contract-supported programs administered by the MBL.

This increase was after a $91,171 decrease in restricted plant expenditures reflecting both the $125,718 expended in 1975 for the landscaping of the Quadrangle with funds provided by the MBL Associates and the expenditure of $30,000 in 1976 to renovate the Neurobiology class laboratory.

Noteworthy also is an increase in 1976 of $50,392 (from $108,590 to $158,982) in scholarships and stipends. This increase reflects the Josiah Macy Foundation grants, the higher level of awards under the Steps Toward Independence Program and the awards to students by The Ecosystems Center.

The increase in the overhead rate and other steps taken to improve income result in a more favorable outlook for the Laboratory than existed a year ago. However, further improvement is needed not only to keep pace with inflation but more importantly to support in full the programs for which the Laboratory is deservedly famous.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

> We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1976, and the related statements of current funds revenues, expenditures, and other changes and changes in fund balances for the year then ended. Our examination was made in accordance with generally accepted auditing standards and, accordingly, included confirmation from the custodians of securities owned at December 31, 1976, and such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.
>
> As more fully described in Note B to the financial statements, the Laboratory excludes certain costs of buildings and equipment from the balance sheet. In our opinion, generally accepted accounting principles require that such costs be included as investment in plant in the financial statements.
>
> In our opinion, except for the effects on the financial statements of the matter discussed in the preceding paragraph, the aforementioned financial

statements present fairly the financial position of Marine Biological Laboratory at December 31, 1976, and its current funds revenues, expenditures and other changes and the changes in its fund balances for the year then ended, in conformity with generally accepted accounting principles applied on a basis consistent with that of the preceding year.

The summary of investments included herein was obtained from the Laboratory's records in the course of our examination and, in our opinion, is fairly stated in all material respects in relation to the basic financial statements taken as a whole.

Boston, Massachusetts
April 1, 1977

Coopers & Lybrand

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1976 and 1975

ASSETS	*1976*	*1975*
Current Funds:		
Unrestricted:		
Cash, including deposits at interest	$ 213,950	$ 49,066
Accounts receivable, net of allowance for uncollectible accounts of $15,566 in 1976 and $20,000 in 1975	446,741	292,477
Other assets	6,141	5,927
Due to restricted current funds	(124,371)	(231,283)
Due from invested funds	65,557	68,505
Total unrestricted	608,018	184,692
Restricted:		
Cash	1,959	37,909
Investments, at cost; market value: 1976—$1,339,892; 1975—$1,169, 931 (Note A, Schedule I)	1,305,084	1,170,009
Due from unrestricted current fund	124,371	231,283
Due from invested funds	136,124	—
Total restricted	1,567,538	1,439,201
Total current funds	$ 2,175,556	$ 1,623,893
Invested Funds:		
Cash	3,297	23,571
Accounts receivable	1,435	—
Investments, at cost; market value: 1976—$4,482, 948; 1975—3,880,277 (Note A, Schedule I)	4,092,770	3,852,003
Due to unrestricted current fund	(65,557)	(68,505)
Due to restricted current funds	(136,124)	—
Total invested funds	$ 3,895,821	$ 3,807,069
Plant Fund:		
Land, buildings and equipment at cost (Note B)	12,345,587	12,378,202
Less accumulated depreciation	3,497,254	3,248,932
Total plant fund	$ 8,848,333	$ 9,129,270

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Balance Sheet

December 31, 1976 and 1975

LIABILITIES AND FUND BALANCES	*1976*	*1975*
Current Funds:		
Unrestricted:		
Accounts payable and accrued expenses	$ 173,387	$ 96,396
Deferred income	60,335	49,037
Fund balance	374,296	39,259
Total unrestricted	608,018	184,692
Restricted:		
Fund balances:		
Unexpended gifts and grants	1,507,577	1,387,044
Unexpended income (Note E)	59,961	52,157
Total restricted	1,567,538	1,439,201
Total current funds	$2,175,556	$1,623,893
Invested Funds:		
Endowment funds (Note E)	2,147,067	1,905,920
Quasi-endowment funds	1,260,509	1,462,894
Retirement fund (Note C)	488,245	438,255
Total invested funds	$3,895,821	$3,807,069
Plant Fund:		
Invested in plant	8,848,333	9,129,270
Total plant fund	$8,848,333	$9,129,270

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Statement of Current Funds Revenues, Expenditures, and Other Changes

for the year ended December 31, 1976

(with comparative summary statement totals for the preceding year)

	Unrestricted	*Restricted*	*1976 Total*	*1975 Total*
Revenues:				
Instruction:				
Tuition	$ 129,605	$ 43,100	$ 172,705	$ 135,400
Grants and contracts:				
Government	5,394	125,301	130,695	242,549
Private		26,916	26,916	91,923
Research (Note D):				
Laboratory rentals	483,952		483,952	371,523
Grants and contracts:				
Government	192,101	373,454	565,555	211,441
Private	132,115	473,181	605,296	379,069
Dormitory	245,349		245,349	258,788
Dining hall	38,985		38,985	150,775
Library	120,318		120,318	92,851
Biological Bulletin	71,727		71,727	70,670
Support departments:				
Research services	138,771		138,771	87,712
Supply	80,104		80,104	93,488
Investment income	147,625	28,016*	175,641*	178,172*
Gifts	96,185	105,834	202,019	218,849
Other	38,291		38,291	35,341
Total revenues	1,920,522	1,175,802	3,096,324	2,618,551

* Investment income from restricted funds is included only to the extent of its application to expenses. Total investment income on unrestricted and restricted funds including the retirement fund was $302,322 in 1976 and $261,540 in 1975 (see Schedule I).

	Unrestricted	Restricted	*1976* Total	*1975* Total
Operating expenditures:				
Instruction	86,351	109,347	195,698	312,332
Research	5,029	846,635	851,664	511,736
Scholarships and stipends		158,982	158,982	108,590
Dormitory	111,590		111,590	112,859
Dining hall	36,300		36,300	134,053
Library	185,669	19,075	204,744	189,990
Biological Bulletin	79,625		79,625	112,143
Support departments:				
Research services	243,891	2,500	246,391	205,529
Supply	197,919	45	197,964	225,404
Administration	398,868	4,671	403,539	397,569
Plant operation	469,083	34,547	503,630	626,919
Other				370
Total expenditures	1,814,325	1,175,802	2,990,127	2,937,494
Excess (deficit) of revenues	106,197	—	106,197	(318,943)
Transfers and additions:				
Excess of restricted gifts and grants received over amounts expended		128,337	128,337	731,275
From plant fund—proceeds of sale of plant assets	29,040		29,040	75,000
From quasi-endowment fund	199,800		199,800	—
Utilized in current operations			—	267,320
Net transfers and additions	228,840	128,337	357,177	1,073,595
Net increase in fund balances	$ 335,037	$ 128,337	$ 463,374	$ 754,652

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Statement of Changes in Fund Balances

for the year ended December 31, 1976

	Current Funds		Invested Funds			
	Unrestricted	*Restricted*	*Endowment*	*Quasi-Endowment*	*Retirement*	*Plant Fund*
Revenues and other additions:						
Unrestricted current fund revenues	$1,920,522					
Grants and gifts		$1,453,746	$ 62,830			
Realized net gains (losses) on sale of investments			178,317	$ (2,585)		
Investment income		136,903			$ 17,794	
Proceeds of sale of equipment						$ 29,040
Addition to pension fund					52,136	
Tuition		43,100				
Total revenues and other additions	1,920,522	1,633,749	241,147	(2,585)	69,930	29,040
Expenditures and other reductions:						
Instruction, research and general expenditures	1,814,325	1,175,802				
Book value of equipment sold						386
Indirect costs		329,610				
Payments to pensioners					19,940	
Depreciation						280,551
Total expenditures and other reductions	1,814,325	1,505,412			19,940	280,937
Transfers among funds—additions (deductions):						
Proceeds of sale of equipment	29,040					(29,040)
Transfer to current funds	199,800			(199,800)		
Total transfers	228,840			(199,800)		(29,040)
Net increase (decrease) for the year	335,037	128,337	241,147	(202,385)	49,990	(280,937)
Fund balances at beginning of year, restated (Note E)	39,259	1,439,201	1,905,920	1,462,894	438,255	9,129,270
Fund balances at end of year	$ 374,296	$1,567,538	$2,147,067	$1,260,509	$488,245	$8,848,333

The accompanying notes are an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

NOTES TO FINANCIAL STATEMENTS

A. *Significant Accounting Policies:*

Basis of Presentation—Fund Accounting

In order to ensure observance of limitations and restrictions placed on the use of resources available to the Laboratory, the accounts of the Laboratory are maintained in accordance with the principles of "fund accounting." This is the procedure by which resources are classified into separate funds in accordance with activities or objectives specified. In the accompanying financial statements, funds that have similar characteristics have been combined.

Externally restricted fund may only be utilized in accordance with the purposes established by the source of such funds. However, the Laboratory retains full control over the utilization of unrestricted funds. Restricted gifts, grants, and other restricted resources are accounted for in the appropriate restricted funds. Restricted current funds are reported as revenue when expended for current operating purposes. Unrestricted revenue is reported as revenue in the unrestricted current fund when received.

Endowment funds are subject to restrictions requiring that the principal be invested and only the income utilized. Quasi-endowment funds have been established by the Laboratory for the same purposes as endowment funds, however, any portion of these funds may be expended.

Investments

Investments purchased by the Laboratory are carried at cost. Investments donated to the Laboratory are carried at fair market value at date received. For determination of gain or loss upon disposal, cost is determined based on the specific identification method.

Investment Income and Distribution

The Laboratory follows the accrual basis of accounting except that investment income is recorded on a cash basis. The difference between such basis and the accrual basis does not have a material effect on the determination of investment income earned on a year-to-year basis.

Investment income includes income from the investments of specific funds and from the pooled investment account. Income from the pooled investment account is distributed to the participating funds on the basis of the market value at the beginning of the quarter, adjusted for the cost of any additions or disposals during the quarter.

Indirect Cost Recovery

The Laboratory recovers indirect costs by charging occupants of laboratory space fees based on an estimated indirect cost rate for the period the space is occupied. The estimated rate is adjusted on a retroactive basis upon determination of actual indirect costs for the year.

B. *Land, Buildings and Equipment:*

Following is a summary of the plant fund assets:

Classification	*1976*	*1975*
Land	$ 639,693	$ 639,693
Buildings	10,143,088	10,148,461
Equipment	1,562,806	1,590,048
	12,345,587	12,378,202
Less accumulated depreciation	3,497,254	3,248,932
	$ 8,848,333	$ 9,129,270

The original cost of land, buildings and related initial furnishing equipment is capitalized when the assets are acquired. The cost of subsequent additions and purchases, repairs and remodeling is expensed when incurred. Equipment and remodeling expenditures amount to approximately $52,000 and $71,000 in 1976 and 1975, respectively.

Depreciation is computed using the straight-line method over estimated useful lives of 40 years for buildings and 20 years for equipment.

C. *Retirement Fund:*

The Laboratory has a noncontributory pension plan for substantially all full-time employees which complies with the requirements of the Employee Retirement Income Security Act of 1974. The actuarially determined pension expenses charged to operations in 1976 and 1975 were $54,973 and $43,353, respectively. The Laboratory's policy is to fund pension costs accrued.

D. *Research Revenues:*

The 1976 research revenues include approximately $111,000 of retroactive overhead rate adjustments to amounts previously billed in 1975.

E. *Reclassification of Endowment Fund Principal and Unexpended Income*:

It was determined that $95,213 of principal in the endowment funds and $22,010 of unexpended income in the current restricted funds balances at January 1, 1975 actually represented unrestricted amounts which should have been included in current unrestricted funds. These amounts have been treated as a correction of the current unrestricted fund balance as of January 1, 1975.

F. *Pledges and Grants:*

As of December 31, 1976, approximately $1,280,000 remains to be received from previous gifts and grants for specific research and instruction programs, and is expected to be received as follows:

1977	$ 891,000
1978	269,000
1979	114,000
1980	6,000
	$1,280,000

SCHEDULE I

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1976

	Cost		*Market*		*1976 Investment Income*
	Amount	*%*	*Amount*	*%*	
Invested Funds:					
U. S. Government securities	$ 603,324	14.7	626,116	14.0	$ 17,714
Corporate bonds	914,634	22.4	801,032	17.9	52,191
Common stocks	2,112,140	51.6	2,597,619	57.9	112,465
Commercial paper	326,367	8.0	326,367*	7.3	21,823
Preferred stocks	118,756	2.9	114,265	2.5	5,229
Real estate	17,549	.4	17,549*	.4	
Total	$4,092,770	100.0	$4,482,948	100.0	209,422
Less custodian fees					10,760
					198,662
Current Restricted Funds:					
U. S. Government securities	1,297,254	99.4	1,332,062	99.4	
Certificate of deposit	7,830	.6	7,830*	.6	
Total	$1,305,084	100.0	$1,339,892	100.0	99,126
Current Unrestricted Funds:					
Earned on corporate savings account					4,534
Net investment income					$302,322
Disposition of investment income:					
Unrestricted-utilized in current operations					147,625
Restricted for current use:					
Research	$ 101,985				
Scholarships	17,067				
Library	17,851				136,903
Retirement fund					17,794
					$302,322

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* At cost.

Reference: *Biol. Bull.*, 153: 98–105. (August, 1977)

THE EMBRYONIC DEVELOPMENT OF THE MARINE CADDIS FLY, *PHILANISUS PLEBEIUS* WALKER (TRICHOPTERA: CHATHAMIDAE)

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Among the few species of insects which spend all or part of their life cycle in marine habitats are certain caddis flies. These trichopterans comprise a single family, the Chathamidae. Adult chathamids inhabit coastal vegetation and their larvae live and develop in intertidal rock pools (Mosely and Kimmins, 1953; Riek, 1970, 1976). The family, which includes four species, is confined mainly to New Zealand and adjacent islands (Chatham Islands, Kermadec Islands), but one species, *Philanisus plebeius* Walker occurs both in New Zealand and along the coast of New South Wales (Riek, 1976). The larvae of *P. plebeius* are well known from rock pools in both localities. The adult female of *P. plebeius*, which has a strong pointed ovipositor, was thought to lay its eggs among coralline algae at periods of low tide (Riek, 1970, 1976). Anderson, Fletcher and Lawson-Kerr (1976), however, have recently shown that the ovipositor is used to insert the eggs into the coelom of one of the arms of a starfish. The host species for the eggs of *Philanisus plebeius* at Cape Banks, N.S.W., is the starfish *Patiriella exigua* Lamarck. The embryonic development of the caddis fly is completed within the starfish coelom, with escape to a free life as a first instar caddis larva. The present paper describes the embryonic development of *Philanisus plebeius* and the seasonal occurrence of oviposition in the host starfish population.

MATERIALS AND METHODS

Thirty large specimens of *Patiriella exigua* were collected at monthly intervals from January to December, 1976, on the intertidal rock platform at Cape Banks, Botany Bay, N.S.W. In the laboratory, the starfish were opened by removal of the aboral body wall, and the coelomic cavities of the arms were inspected for the presence of *Philanisus* eggs (Fig. 1). The sex of the starfish was also noted. Batches of *Philanisus* eggs obtained in this manner were treated in two ways. After staging by direct observation of the state of development of the living embryos, the majority of egg masses were fixed in Kahle's fluid (formalin: alcohol: acetic acid, 6: 16: 1). Some batches of eggs were transferred to Petri dishes of sea water and maintained at 23–25° C, the water being changed every two days. In these culture conditions, the embryos continued to develop normally, allowing the timing of development and the external changes in the living embryo to be recorded.

Fixed embryos of different stages were dehydrated through methyl benzoate and benzene after piercing the chorion with a fine needle. The cleared embryos were then mounted unstained in Eukitt and used to elucidate further details of external structure at each stage.

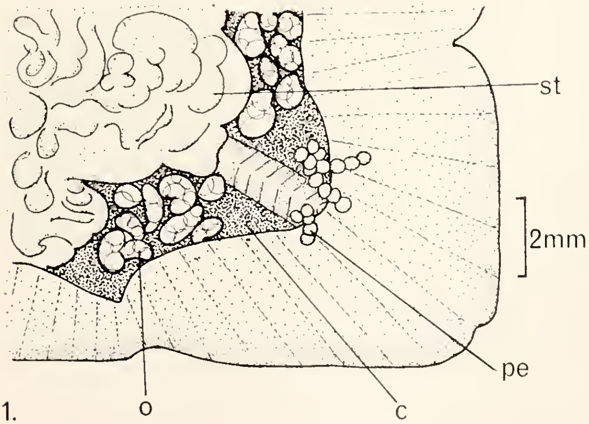
RESULTS

Seasonal occurrence

Embryos of *Philanisus plebeius* were obtained from starfish hosts in every month of the year except the winter months of June and July, and the mid-summer month, January. The numbers of host starfish in each monthly sample of 30 individuals were as follows: January, 0; February, 6; March, 8; April, 2; May, 6; June, 0; July, 0; August, 2; September, 8; October, 8; November, 8; December, 1. In the majority of hosts, only one batch of eggs was found in each host individual. The eggs of a batch adhered loosely together and showed synchronous development, indicating that they had resulted from a single oviposition. The arm selected for oviposition was random, showing no fixed relationship with the madreporite and no discrimination of the sex of the host. In a few host individuals, two batches of eggs were found in different arms, one batch being advanced in development, the other early, clearly the result of separate ovipositions. None of the starfish examined were found to contain hatched, first instar larvae of *Philanisus,* although on several occasions hatching took place shortly after the host had been opened. The newly hatched larvae were very active and voracious, attacking the dissected host tissue. It seems likely that they eat their way out through the host body wall immediately after normal hatching.

Embryonic development

In each batch of eggs, 30–50 eggs are grouped together in short strings (Fig. 2), adhering loosely by chorionic contact. When transferred to sea water in a Petri dish, the individual eggs of a batch can be separated by light pressure with a fine dissecting needle.



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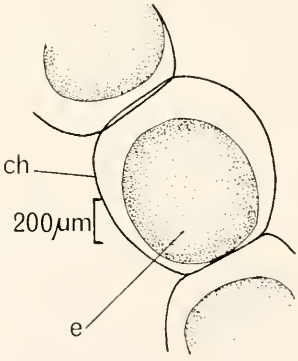

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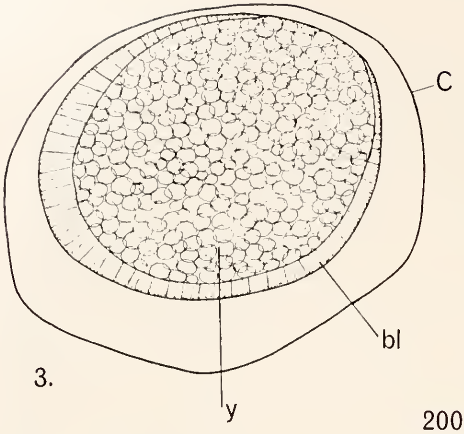
FIGURE 1. The eggs of the marine caddis fly, *Philanisus plebeius*, exposed by dissection of the oviposition host, the starfish *Patiriella exigua*. Abbreviations are: *c,* coelom; *o,* ovary; *pe,* egg of *Philanisus;* and *st,* stomach.

FIGURE 2. Part of an egg string of *Philanisus plebeius*. Abbreviations are: *ch,* chorion; and *e,* egg.



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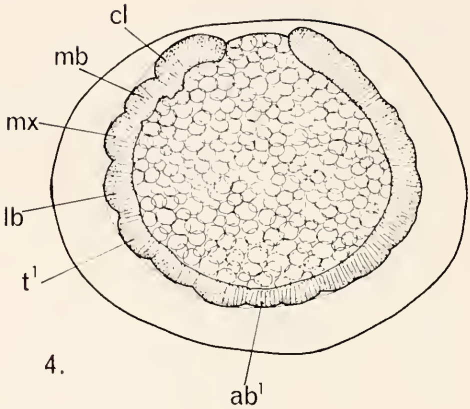
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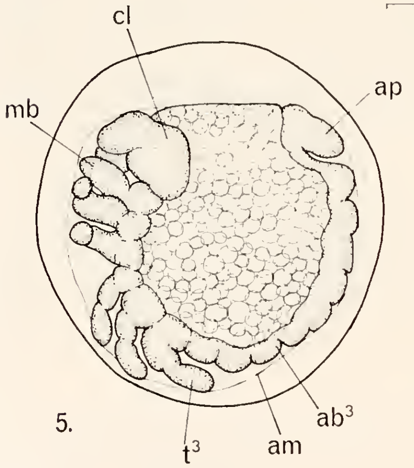
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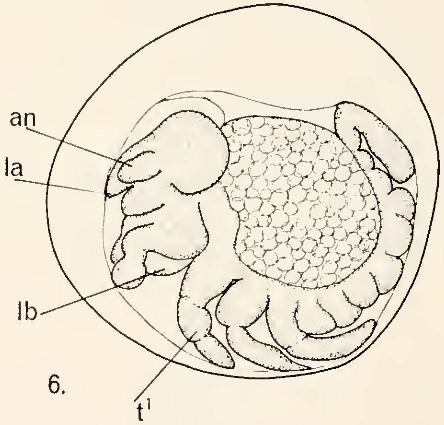

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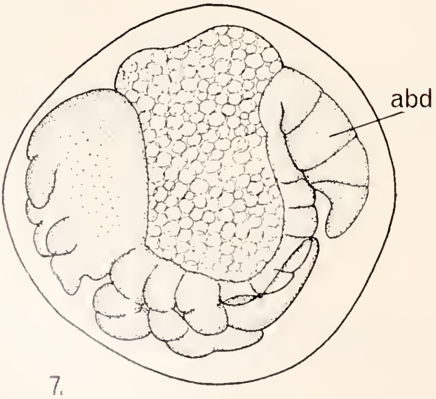

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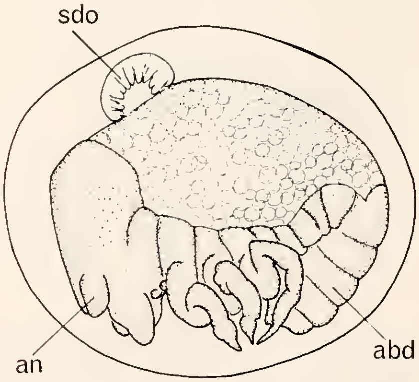

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In eggs observed shortly after oviposition, when the embryos are still at the stage of forming a germ band, each egg is ovoid, yellowish and densely yolky, with a long diameter of 0.27 mm. The transparent chorion which surrounds the egg is, in contrast, almost spherical and has a diameter of 0.39 mm. As development proceeds, the embryo enlarges to fill the space within the chorion. The chorionic diameter increases slightly before hatching takes place.

During the first three days of development, a blastoderm is formed at the yolk surface (Fig. 3), the embryonic primordium gradually differentiated, and an elongate, segmenting germ band is formed (Fig. 4). The segmenting germ band extends around most of the circumference of the egg along the ventral midline, with the end of the abdominal rudiment approaching the cephalic lobes. The mouthpart segments, thoracic segments and first few abdominal segments can be distinguished at this stage.

During the fourth to sixth days of development, the remaining abdominal segments become delineated, the cephalic lobes enlarge, antennal rudiments form and limb buds develop on the mouthpart and thoracic segments (Fig. 5). A pair of large rudiments of prolegs also forms on the tenth abdominal segment. On the seventh day, these limb buds increase further in length and begin to show podomere delineation. The mouthparts become more closely grouped together, while the thoracic limbs extend in a posterior direction beneath the ventral surface of the embryo, which retains its convex curvature (Fig. 6).

The embryo now performs a blastokinetic movement which reverses its curvature, from ventrally convex to ventrally concave, and is accompanied in its later phases by dorsal closure. This movement takes place through the eighth to ninth days. The first sign of blastokinesis is a downturning, tubulation, and forward thrust of the posterior end of the abdomen (Fig. 7). This movement proceeds until the entire abdomen is ventrally flexed (Figs. 8, 9), the yolk mass being now confined to the thorax and anterior part of the abdomen. At the same time, the bases of the thoracic limbs are shifted to a more lateral position on their respective segments. The stomodaeum and the elongating proctodaeum also become conspicuous during blastokinesis.

By the time the blastokinetic movement is complete (Fig. 10), the posterior end of the ventrally flexed abdomen is in contact with the head. Dorsal closure is complete, the remaining yolk is confined to the anterior part of the abdomen and secretion of the cuticle has begun. The remainder of the development of the embryo, from 10 days to hatching at 17–18 days is completed in this position

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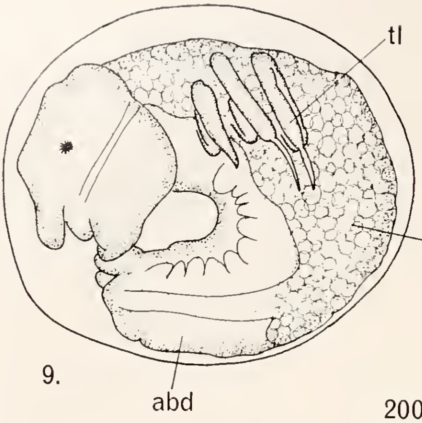
FIGURE 3. *Philanisus plebeius,* blastoderm stage. Abbreviations are: *bl,* blastoderm; *c,* chorion; and *y,* yolk.

FIGURE 4. *Philanisus plebeius,* segmenting germ band stage. Abbreviations are: *ab*^{1}, first abdominal segment; *cl,* cephalic lobe; *lb,* labium; *mb,* mandible; *mx,* maxilla; and *t*^{1}, first thoracic segment.

FIGURE 5. *Philanisus, plebeius,* early limb bud stage, 6 days. Abbreviations are: *ab*^{3}, third abdominal segment; *am,* amnion; *ap,* anal proleg; *cl,* cephalic lobe; *mb,* mandible; and *t*^{3}, third thoracic segment.

FIGURE 6. *Philanisus plebeius,* embryo approaching blastokinesis, 7 days. Abbreviations are: *an,* antenna; *la,* labrum; *lb,* labium; and *t*^{1}, first thoracic segment.

FIGURES 7 and 8. *Philanisus plebeius,* early stages in blastokinesis, 8 days. Abbreviations are: *abd,* abdomen; *an,* antenna; and *sdo,* serosal dorsal organ.



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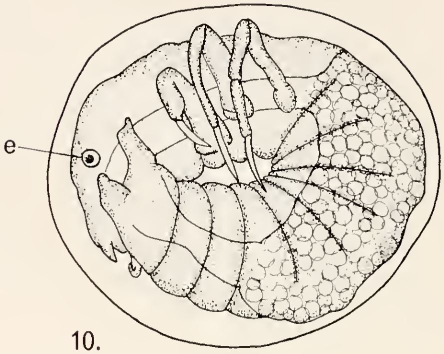
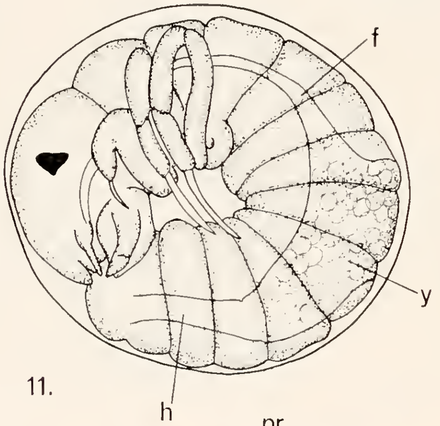


10.

200μm



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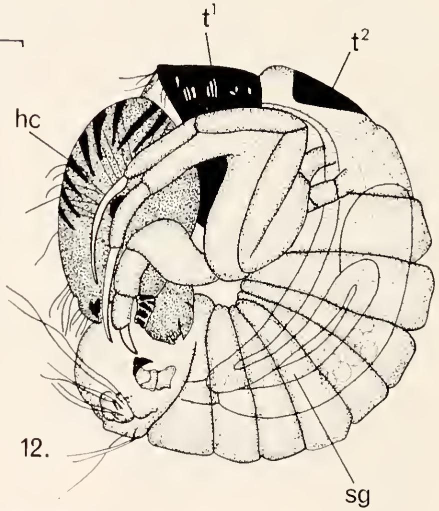

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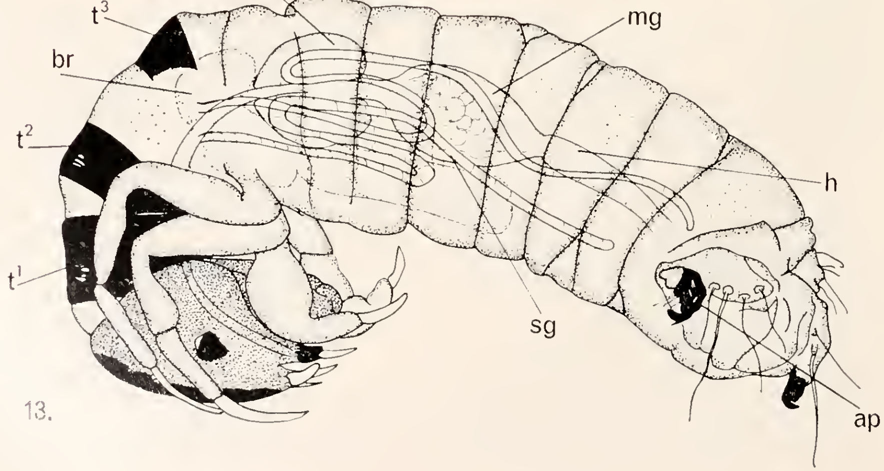
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13.

(Figs. 11, 12). The yolk reserves are gradually resorbed, only a small remnant persisting in the midgut when the larva hatches. Cuticularization of the head capsule, the first and second thoracic terga, the limbs and the abdominal prolegs becomes especially conspicuous during this period.

At hatching, the emerging larva (Fig. 13) is a typical trichopteran larva in both structure and movements. The mouthparts are functional, the larva crawls actively using the thoracic limbs, and a house is quickly constructed using available materials. If left among the tissues of the dissected host, the larva makes a house from pieces of broken starfish ossicle. Normally, the larva forms its house from pieces of material, mainly coralline alga, obtainable in its rock pool habitat.

Discussion

The present work confirms the fact, first pointed out by Anderson, Fletcher and Lawson-Kerr (1976), that *Philanisus plebeius* oviposits in the coelom of a host starfish, *Patiriella exigua,* and completes its embryonic development in the host coelom before hatching and escaping as a first instar larva.

In the *Philanisus* population of Cape Banks, N.S.W., oviposition occurs during most of the year, except for the winter months June and July and perhaps the midsummer month, January. Oviposition is active during the spring (September to November) and late summer to autumn (February to May). Since the duration of embryonic development is less than three weeks, young larvae might be expected to be present in the rock pools in the spring and autumn.

Riek (1976) provides data on the seasonal occurrence of larvae and pupae of *Philanisus plebeius* at Broulee, N.S.W., which support the concept of two breeding peaks at these times. He found that adults were present and young larvae were abundant during the spring, but that fully grown larvae and pupae predominated in December and Jaunary. Gravid adults then reappeared in February and persisted through the autumn.

It is not known at the present time whether the adults of *P. plebeius* have a short or an extended breeding life, nor how the species overwinters. The occurrence of oviposition in May suggests that a larval population is maintained during the winter months, emerging as adults in the following early spring. Overwintering by adults following emergence in late autumn is also a possibility.

Although the oviposition host of *P. plebeius* in New South Wales is *Patiriella exigua,* it seems likely that another host species of starfish is utilized in New Zealand, since *P. exigua* does not occur there (Anderson, Fletcher and Lawson-Kerr, 1976). The identity of this species remains to be established. The work

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Figure 9. *Philanisus plebeius,* later stage in blastokinesis, 9 days. Abbreviations are: *abd,* abdomen; *tl,* thoracic limb; and *y,* yolk.

Figure 10. *Philanisus plebeius,* blastokinesis and dorsal closure complete, 10 days. Abbreviation is: *e,* eye.

Figure 11. *Philanisus plebeius,* embryo at 13 days. Abbreviations are: *f,* foregut; *h,* hindgut; and *y,* yolk.

Figure 12. *Philanisus plebeius,* embryo at 17 days, with chorion removed. Abbreviations are: *hc,* head capsule; *sg,* salivary gland; and t^1, t^2, thoracic segments.

Figure 13. *Philanisus plebeius,* newly hatched larva, 18 days after oviposition. Abbreviations are: *ap,* anal proleg; *br,* brain; *h,* hindgut; *mg,* midgut; *pr,* proventriculus; *sg,* salivary gland; and t^1, t^2, t^3, thoracic segments.

of Riek (1976) also shows that the females of the other three species of Chathamidae, *Philanisus fasciatus* in the Kermadec Islands, the brachypterous *Chathamia brevipennis* in the Chatham Islands and *Chathamia integripennis* in northern New Zealand, all have a strong pointed ovipositor similar to that of *P. plebeius*. It therefore seems likely that each species is associated with one or more species of starfish as an oviposition host. *C. brevipennis* has a rock pool larva similar to that of *P. plebeius*. The larvae of the other two species have not yet been described.

It is perhaps significant that the oviposition host of *P. plebeius, Patiriella exigua,* has an unusual breeding pattern, in which adults of both sexes maintain a constant state of reproductive maturity throughout the year, with spawning taking place opportunistically from time to time in response to appropriate environmental conditions (Lawson-Kerr and Anderson, unpublished). The coelomic cavity of the host species is thus not subject to the cyclic variation of gonad expansion and reduction that takes place in starfish species with an annual breeding cycle and a limited spawning season. The oviposition hosts of the other chathamid species may or may not show this phenomenon.

In spite of the unusual ovipostion site of *P. plebeius*, the eggs and mode of embryonic development of the species retain the typical trichopteran pattern. The only trichopteran species whose embryonic development has been studied in detail is *Stenopsyche griseipennis,* recently investigated by Miyakawa (1973, 1975). *S. griseipennis* oviposits on rock surfaces in freshwater streams. The egg is larger than that of *P. plebeius,* being 0.56 mm in length, but develops more rapidly, with hatching taking place after 12 days at 16–21° C. Miyakawa describes a remarkable invaginate formation of the embryonic primordium, but no comparison of this stage can be made for *P. plebeius,* due to a lack of critical early stages. Once the segmenting germ band has begun to elongate over the surface of the yolk mass, however, the development of the two species proceeds in a similar manner. The only notable difference associated with the smaller egg size in *P. plebeius,* 0.27 mm in length, is that the segmenting germ band extends further onto the dorsal surface of the yolk mass than in *S. griseipennis*. The middle period of development in both species is characterized by a blastokinesis, accompanied by dorsal closure, in which the curvature of the embryo is reversed within the egg space. This movement occupies three days in *S. griseipennis* and two days in *P. plebeius*. As Miyakawa (1975) points out, the trichopteran blastokinetic movement is a specialized embryonic movement shared with Lepidoptera (*e.g.,* Anderson, 1972; Wall, 1973), but differs from that of Lepidoptera in that the entire yolk mass of the trichopteran embryo is enclosed within the embryo in the usual pterygote manner. In Lepidoptera, much of the yolk mass is left outside the embryo during dorsal closure, to be consumed later through the mouth (Anderson and Wood, 1968).

The embryonic development of *P. plebeius,* therefore, shows no special structural modifications related to its intracoelomic site for development. The fact that the embryos continue to develop normally in sea water also excludes the possibility of any nutritional dependence. Possibly the major adaptive advantage of the oviposition relationship between *P. plebeius* and its starfish host lies in solving the problem of maintaining the eggs in the rock pool environment into which the larvae hatch. The eggs are loosely agglomerated and would, if laid among weed, be subject to wave dislodgement and to dessication during low tide. The starfish coelom provides a stable, protected environment for the embryos and eliminates the possibility of predation. As far as we are aware, no similar oviposition and embryonic development within the body cavity of an intertidal invertebrate host has been reported for any other species of insect. A major unsolved problem is how the newly hatched larvae escape from the host into the rock pool. Some modification of the normally carnivorous habit of a trichopteran larva is presumably involved.

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SUMMARY

1. *P. plebeius,* a trichopteran with marine intertidal larvae, oviposits in the coelom of a starfish, *Patiriella exigua.* Oviposition occurs mainly in the spring and autumn months.

2. In spite of the intracoelomic location of the embryos, the development of *P. plebeius* follows an unmodified trichopteran mode, including the characteristic blastokinesis. Nutrients are not supplied to the caddis embryos by the host starfish.

3. Hatching takes place in the starfish coelom after 17–18 days. The newly hatched caddis larvae quickly escape to their rock pool habitat.

4. The form of the female ovipositor indicates that other species of Chathamidae utilize starfish species as oviposition hosts.

5. This mode of oviposition offers protection to the caddis embryos in the intertidal habitat.

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CAROTENOID PIGMENTS IN THE COXAL GLAND OF *LIMULUS*

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Packard (1875) was the first to call attention to an organ in *Limulus polyphemus* which he stated (p. 511) was "conspicuous from its bright red color." Lankester (1882, p. 95) in his studies on this tissue referred to it as the "brick-red gland." He noted the structural resemblance of this tissue to the colorless coxal glands of *Scorpio*, which led to the development of his much-debated thesis (*cf.*, Versluys and Demoll, 1922) that *Limulus* was to be classed as an arachnid. Following these reports, a number of anatomical studies by Galland (1885) and Patten and Hazen (1900) appeared which provided evidence in support of the view originally expressed by Packard (1875, p. 513) that the red coxal gland of *Limulus* was "renal in its nature." More recent studies with the electron microscope by Karnovsky and Briggs (Harvard Medical School, personal communication) extend the evidence that this tissue has a morphological structure resembling that of a nephridium. Though Packard, over a hundred years ago, described this tissue as conspicuous for its bright red color, more conspicuous today is the apparent absence of any reports in the literature on the chemical identity of its coloring matter. The present investigation was therefore undertaken in an attempt to supply this missing information. Evidence is presented that the red color of the coxal gland of *Limulus* is due to its high content of carotenoids.

Materials and Methods

The coxal gland of *Limulus* is a bilateral organ with a total of eight lobes which lie embedded in the muscles around the base of the second, third, fourth, and fifth legs. In this study large adult animals were employed (carapace width 15–28 cm), and the tissue from all the lobes was trimmed free of muscle and pooled. No attempt was made to effect a quantitative recovery of the tissue, and the total amount obtained varied from 250 to 1350 mg, depending upon the size of the animal. The tissue was ground in an all-glass homogenizer with absolute methanol, the mixture centrifuged, and the supernatant solvent collected. Extraction was repeated until no further pigment was removed, and the nearly colorless residue was discarded. Water was added to the methanol solution to bring its concentration to 90% and extracted with petroleum ether (bp. 30–60° C) until the extracts were colorless. The petroleum ether solution was washed with water to remove methanol and subjected to column chromatography. Columns of an aged aluminum oxide preparation were found most useful for this purpose using petroleum ether with increasing concentrations of acetone to separate and elute the carotenoids. The purity of the various fractions yielded by this procedure was checked and R_F values obtained by thin layer chromatography on silica gel plates using either a 20% acetone-80% hexane or a 20% acetone-80% petroleum ether as a developing solvent. Spectrophotometric determinations were made with a Beckman DU spectrophotometer on which the wavelength setting was checked using the 550 nm band of reduced cytochrome c as a reference point. Organic solvents employed were analytical or spectrophotometric grade. During all procedures an effort was made to exclude light and to keep solutions under a nitrogen atmosphere.

RESULTS

Initial experiments revealed that no pigment could be extracted by homogenization of the tissue with water or 0.5 M NaCl solutions. Examination of the intact tissue or aqueous homogenates with a hand spectroscope revealed no bands characteristic of the hemochromogens, either before or after the addition of the reducing agent dithionite. The bulk of the pigment could be readily extracted with either acetone or methanol. Spectroscopic examination of such extracts revealed a spectrum characteristic of the carotenoids with a peak at 450–453 nm and a shoulder at 475 nm. Dilution with water of a methanol solution to 90% permitted the extraction of over 90% of the pigment by petroleum ether. The pigment remaining in the methonal phase displayed the spectrum of carotenoids and could be extracted into petroleum ether or chloroform upon further dilution of the methanol with water. Further characterization of this pigment was not attempted.

Total carotenoid content

An estimate of the total carotenoid content of the coxal gland was made by measuring the optical density of an extract of the gland at its absorption maximum, usually 450 nm. For this purpose a value of 2500 for $E^{1\%}_{cm}$ was employed. Measurements were made either on the original methanol extract or most often on the petroleum ether extract of the diluted methanol solution. The total carotenoid content obtained in this manner ranged from 25 to 226 μg per gram of wet tissue. The corresponding average value for glands from 21 animals (11 females, 10 males) was 111 μg. Glands which had a brick-red color to the eye contained the highest content of carotenoids. In many cases the intact gland has a yellow color to the eye but reveals a red color upon cross section. In one experiment the exterior yellow portion and the interior redder portion were dissected free from one another. The carotenoid content of the pale exterior portion was 62 μg and that of the interior redder portion 210 μg per gram wet weight. There is some indication that glands with the highest carotenoid content are found in those animals fresh from their native habitat, although this requires further documentation.

Separation and partial identification of carotenoids

The multiple nature of the carotenoids in the coxal gland is clearly revealed by column or thin layer chromatography of a petroleum ether extract. Seven to eight components are clearly recognizable by these procedures. An example of the results to be obtained is illustrated by the data presented in Table I. In this experiment the glands from three animals were used, and 91% of the carotenoids were removed by petroleum ether from the 90% methanol extract. A portion of the petroleum ether extract was added to a column of aluminum oxide, 9 × 1 cm, and developed with petroleum ether containing increasing amounts of acetone, as indicated in Table I. The fractions containing acetone were evaporated to dry-

TABLE I

Relative abundance and chromatographic and spectral characteristics of coxal gland carotenoids.

Fraction	Eluent	Absorption peaks nm	R_F	Percentage of total
1	Petroleum ether	449,475	0.97	8.6
2	1% Acetone*	455	0.55	28.8
3	2% "	455	0.48	7.0
4	5% "	449,475	0.46	3.4
5	5% "	447,474	0.32	12.3
6	10% "	450	0.26	5.5
7	20% "	447	0.22	5.2
				70.8

* Percentage volume of acetone in a petroleum ether-acetone mixture.

ness in the dark under a stream of nitrogen and the residue dissolved in petroleum ether for spectrophotometric and R_F determinations. The percentage of the total recovered in each fraction was determined from the volume and optical density at 450 nm of the fraction, as compared to measurements made on the original petroleum ether extract. The R_F values were determined on silica gel plates developed with a 20% acetone-80% hexane mixture.

As judged by the results of thin layer chromatography, a fairly clean separation of components was obtained in this experiment and nearly all fractions appeared to contain a single component. Additional information and some of the conclusions that may be drawn as to the nature of some of the various components are as follows.

Fraction 1. The absorption spectrum and R_F value of this component are identical with those obtained on an authentic sample of β-carotene, and it would appear that this component is β-carotene.

Fraction 2. Its absorption spectrum is broad and resembles that of a keto-carotenoid such as echinenone. In 95% ethanol, this component displays a broad absorption band centered at 465 nm. Upon reduction with borohydride, two peaks at 452.5 and 480 nm appear. In petroleum ether, the reduced compound exhibits peaks at 447.5 and 475 nm, and its R_F is 0.40. This major component thus appears to be a keto-carotenoid.

Fraction 3. In 95% ethanol the component in this fraction has a broad absorption band centered at 470–475 nm. Upon reduction with borohydride, two peaks appear at 451 and 480 nm. In petroleum ether, the reduced compound shows two peaks at 449 and 475 nm, and its R_F is 0.38. This component thus appears to be another keto-carotenoid, not unlike that in fraction 2.

Fraction 4. The material in this fraction, unlike the others, does not yield a sharp R_F value, and the value given in the table represents the average of an R_F spread from 0.40 to 0.52. There is thus a possibility that more than one component is present in this fraction.

Fraction 5. The absorption spectrum of this fraction very closely resembles that of the reduced form of the component in fraction 2 suggesting that it contains a hydroxycarotenoid. No change in spectrum occurred upon treatment of its ethanolic solution with borohydride.

Fraction 6. The absorption peak at 450 nm of this fraction is unusually distinct with a well-defined shoulder at 475 nm.

Fraction 7. In 95% ethanol, the main absorption of this fraction lies at 450 nm. Upon reduction with borohydride, the position of this absorption band remains unchanged but it is sharpened and increased.

After the collection of the seven fractions listed in Table I, which accounted for 71% of the total material added to the column, there still remained pigment on the column near its top. This material could not be removed by either 100% acetone or methanol. Glacial acetic acid did remove most of this material, but further characterization of it has not proved feasible.

It should be emphasized that although there is some similarity in the results obtained from experiment to experiment, noticeable differences are encountered. For example in another run the material in fraction 1, presumably β-carotene, accounted for about 20% rather than 8% of the total. Also in this experiment, fraction 2 represented about 32% of the total, while the total amount recovered from the column was about 85%. Thus, both the total carotenoids present as well as the composition can vary from animal to animal.

Carotenoids in other tissues of Limulus

A less extensive examination of the carotenoids of three other *Limulus* tissues was made so that some comparison could be made with those found in the coxal gland. The extraction of these tissues and the carotenoid measurements were carried out in the same manner as described for the coxal gland. The values given here for these tissues are based upon the examination of a petroleum ether solution obtained by extraction of the original methanol extract after its dilution to 90% with water. On this basis, the hepatopancreas of four animals yielded total carotenoid contents of 30, 32, 15, and 48 μg per gram wet weight, for an average value of 31 μg. Eggs obtained from two animals yielded total carotenoid contents of 8.6 and 15.5 μg per gram wet weight. Analysis of the total blood clot obtained from two animals gave values for total carotenoids of 1.7 and 3.3 μg per gram wet weight. Expressed in terms of the amount present in 100 ml of whole blood these values are 6.1 and 9.7 μg, respectively. In one experiment the amebocytes of the blood were isolated by the technique of Murer, Levin, and Holme (1975). The total content of carotenoids in these cells was 5.7 μg per ml as packed by centrifugation. Since 100 ml of whole blood yielded 1.35 ml of cells, the corresponding value for 100 ml of whole blood is 7.7 μg.

In the case of each tissue the petroleum ether extracts were subjected to thin layer chromatography. Based upon R_F values observed in these experiments, the mixture of carotenoids in the eggs and amebocytes resembled most closely those present in the coxal glands. The three chief carotenoids present in these tissues appeared to be the same as those found in the coxal gland, namely those listed as fractions 1, 2, and 5 in Table I. The mixture of carotenoids present in the hepatopancreas bore the least resemblance to those in the coxal gland, with β carotene (fraction 1, Table I) being the only component that was clearly identifiable. It may also be noted in the case of the hepatopancreas that as measured by total absorption at 450 nm, only about 50% of the pigment in the 90% methanol extracts is transferable to petroleum ether. The corresponding value for the other tissues including the coxal gland is close to 90%.

Discussion

The brick-red color of the coxal gland of *Limulus* noted by earlier investigators is undoubtedly to be attributed to its high content of carotenoids. The average carotenoid content of the glands from 21 animals was 111 μg per gram wet weight, while the highest value observed was 226 μg. A table of the carotenoid content of marine invertebrates is given by Goodwin (1954). The highest value to be found in this table is 149.6 μg per gram wet weight for the coelenterate, *Metridium senile* (red). Examination of three other tissues of *Limulus* (eggs, blood, and hepatopancreas) revealed that the highest carotenoid content of these three tissues was in the hepatopancreas, which had an average value of 31 μg per gram wet weight. The concentration of carotenoids in the coxal gland is thus much greater than in any of the other tissues that were examined.

It is generally agreed that animals are not capable of the *de novo* synthesis of carotenoids. Their occurrence in animals is therefore to be attributed to dietary intake and the ultimate source is to be traced back to plants in the food chain. Thus, the wide variation in the concentration of carotenoids in the coxal gland reported here, 25–226 μg per gram wet weight, may well represent the previous dietary history of the animals. The specimens of *Limulus* used in this study were not only collected at different seasons of the year and locations, but some were also held unfed in captivity for varying and unknown lengths of time. No correlation between the sex of the animal and the content of carotenoids in the coxal gland was discernible.

The reason for the accumulation of carotenoids in the coxal gland is not clear. Since what evidence is available suggests that this tissue is renal in nature, one premise that may be drawn is that during its excretion of waste products the carotenoids are retained by this tissue for some unknown reason. The possibility that these pigments play some role in the function of the gland can not be ruled out at this time. Indeed, any consideration of this possibility must await a fuller understanding of the functions of the coxal gland. Most of the earlier studies are morphological in nature, and very few reports are to be found on the excretions of this tissue. Tower (1895, p. 472) in his description of the external opening of the coxal gland wrote, "I have many times noticed a white transparent fluid oozing to the exterior through the external openings. Chemical analyses which are now being carried on indicate that the glands are of an active excretory nature." A search of the literature has failed to reveal any subsequent publication of Tower's chemical analyses. More recently, Mangum, Booth, DeFur, Heckel, Oglesby, and Polites (1976) have reported on Na^+, Cl^-, NH^+_4 concentrations, pH values and total solids in excretions of the coxal gland of *Limulus*. Their data indicate that the concentration of sodium and chloride ions and the total osmolality of the urine and blood are similar for *Limulus* inhabiting waters with the salinity of normal sea water. Concentrations of NH^+_4 in the urine are some eight times higher than in the blood, and the urine is more acid than the blood. Subjecting *Limulus* to water of low salinities results in a lowering of the osmolality of both blood and urine. Even though rates of urine secretion are not given, these results suggest that the coxal gland is not required to expend much energy in its treatment of the inorganic components of the animal.

It seemed logical to conclude that the immediate source of the carotenoids of the coxal gland was to be found in the blood of *Limulus*. Since the blood clot has a yellowish color, attention was focused on it and the amebocytes that it contains, rather than on the clot-free portion containing hemocyanin. Carotenoids were found to be present in both the whole blood clot and the isolated amebocytes though in concentrations which are relatively small when compared to those of the coxal gland. It is of interest that, as revealed by thin layer chromatography, the mixture of carotenoids found in this fraction of the blood bears a closer resemblance to those in the coxal gland than those present in the hepatopancreas. The amebocytes contain granules, and Murer, Levin, and Holme (1975) have presented evidence that these granules contain all the factors required for the coagulation of the blood. According to Lankester (1884, p. 161) the color of the coxal gland "is due to the presence of numerous small red-coloured granules which occur in the layer of gland-epithelium." It would be of interest to know whether these granules or their contents are derived from the granules of the amebocytes.

An abundant source of both the blood and coxal gland carotenoids could be the hepatopancreas, which is a very large organ in *Limulus* and contains on the average 31 μg of carotenoids per gram wet weight. However, the carotenoids of the hepatopancreas display properties which indicate that they are different from those found in the coxal gland or blood. Hence, metabolic conversions of the hepatopancreas carotenoids must occur if they are the source of those present in the blood and coxal gland. If such conversions occur, the site is not indicated by the studies presented here.

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SUMMARY

The coxal gland of *Limulus polyphemus* contains a high content of carotenoids. Values ranging from 25 to 226 μg per gram of wet tissue were found. The corresponding average value for 21 animals was 111 μg. Chromatographic separation reveals the presence of 7 to 8 components. Characterization and partial identification of these components has been made by the determination of R_F values and absorption spectra. In some cases the alterations produced in these properties by borohydride reduction have also been presented. The brick-red color of this tissue reported by earlier investigators would appear to be largely due to its high content of carotenoids.

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DWARFISM IN A SABELLID POLYCHAETE, A STUDY OF AN INTERSTITIAL SPECIES

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Sabellid polychaetes are tubicolous worms that feed by means of a crown of tentacles ordinarily extending from the distal end of a mud-encrusted, parchment or mucous tube. The numerous species together exhibit a remarkable range in size, from relatively giant forms such as *Eudistylia vancouveri* (up to 30 cm long and a centimeter wide) to semi-microscopic species barely visible to the naked eye. The present account is of a species of the subfamily Fabricinae, so small as to be truly microscopic, which has an interstitial detrital habitat. Its interest is that the species apparently represents the ultimate degree of dwarfism possible, and that since live individuals are virtually transparent, they elegantly display their internal structure and activity. Previous accounts of internal structure have been based on dissected and sectioned specimens of larger kinds (Nicol, 1930).

Material and Methods

The material studied was found in detritus accumulating along the shaded sides of wood floats in a small protected lagoon of Coconut Island in Kaneohe Bay, Oahu, Hawaii. Specimens were found only by means of a low-power microscope and by virtue of their characteristic thrashing movements when disturbed. The species may have a wide distribution but without such movement individuals are not likely to be seen, the local agitation of detritus particles being the most obvious sign of their presence. The overall length of a mature individual, including tentacles, is about 2.5 mm maximum. Body length exclusive of tentacles is about 1.8 mm. Juveniles of much smaller size are usually present.

Results

Structure and function

The organization and activities of this small sabellid are basically the same as in the largest. They relate primarily to feeding by means of the tentacular crown when protruded from the anterior end of the tube, to the formation of the tube itself, and to behavior within the tube. Inasmuch as individuals abandon the tube upon any disturbance, the subsequent explorative activity is also important in this species. The three main divisions of the worm are the head (tentacle and collar region), the thorax, and the abdomen (Fig. 1A).

When at rest, the worm lies with the prostomial crown of tentacles extending entirely beyond the distal end of the tube. The crown consists of a distinct right and left unit, each consisting of three radioles bearing seven or eight pairs of long lateral filaments, or pinnules, which extend to the crown perimeter (Fig. 1B).

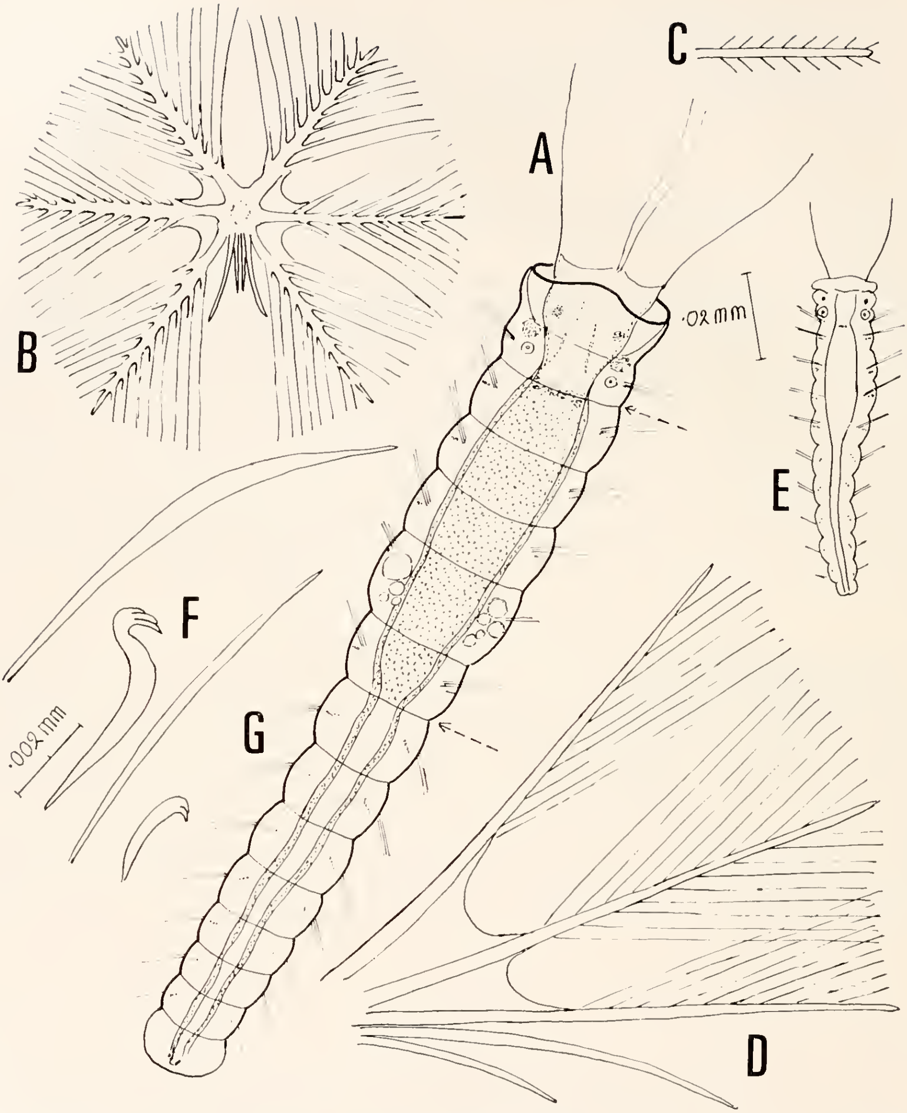

FIGURE 1. General structure of an interstitial sabellid species: A, full grown female individual at onset of sexual maturity; gonads, male or female, develop only in the last but one thoracic segment; B, crown of tentacles seen from anterior end, showing three pinnule-bearing radioles together with two simple tentacles on each side, and mouth at center; C, a pinnule bearing stiff hairs; D, side view of a lateral tentacular complex with the three tentacular radioles bearing long pinnules, a web uniting the basal part of the radioles, and the pair of abortive, explorative tentacles; E, a juvenile, to same scale as in A; F, seta and long-handled hook typical of thoracic segments; and G, seta and hook of abdominal segments.

The pinnules are ciliated and draw water through the external side of the crown and forward through the crown opening, while radiolar cilia cause trapped, microscopic particles to move basally toward the ciliated entrance to the mouth, as in *Sabella pavonina* (Nicol, 1930). Ciliary activity is evident when the crown is expanded but ceases when the radioles close together, *i.e.*, when the worm withdraws into its tube or is actively travelling backward outside the tube. The pinnules also bear stiff pointed hairs (Fig. 1C) which appear to be offensive but not toxic to the larger ciliates coming in contact with them. In addition to the tentacular complex just described, two additional radioles on each side, lacking pinnules and relatively small, serve as explorative tentacles when the worm is free of its tube and moving forward (Fig. 1B, D).

The base of the crown on each side exhibits a groove (Figs. 1A, 2A), which in larger species indicates the line of autotomy, allowing sacrifice of the crown and survival of the remainder of the individual when pulled by a predator. In the present species such a hazard may not exist, and the autotomy groove may be a relict persisting in spite of an extreme degree of dwarfism.

The collar segment, next to the prostomial, tentacular crown, is comparatively simple, the collar rim being undivided, not developed into folds, as in most sabellids. In larger forms the collar is mainly responsible for adding detrital and other materials to the growing distal end of the mucous tube. In this species the collar and related features are so small, relative to the size of the particles available for tube reinforcement, that such collar activity is not feasible. Detritus particles, however, do adhere to the sticky external surface of the delicate mucous tube throughout its length.

The tube of mucus, open at both ends, is quickly formed wherever and whenever the individual comes to rest. While the crown is generally protruded anteriorly for feeding and respiration, the worm periodically withdraws completely and can reverse its position within the tube in spite of close confinement. Any disturbance, however, and any direct exposure to a source of light, causes the worm to wriggle backward from the tube and to progress in a straight line, leaving a trail of mucus behind it. As it does so, it intermittently sweeps the posterior half of its body through nearly 180 degrees in an exploratory manner, an activity that persists until contact with dense detritus and comparative shade is encountered. Action then ceases, a new tube is formed within a few minutes, the crown is protruded, and ciliary currents are re-established. The animal may also advance forward, at which time the pair of simple radiole tentacles on each side explore any substrate within reach.

In a number of sabellid species the posterior terminal structure, the pygidium, bears a pair or several eyespots, particularly among the smaller species of the Fabricinae (*e.g.*, *Fabricia sabella*). The activity just described suggests that pygidial light-sensitivity exists even in the absence of such visible ocelli. Pigment in the present species is restricted to the pair of larval eyes persisting in the cerebral ganglia of sabellids (Wilson, 1936; Downer, 1961), none being present on the body or tentacles. The relative transparency, however, exposes the cephalic ocelli to light even in fully mature individuals, whereas in large individuals of other species, tissue density tends to obscure the ocelli.

A pair of otocysts (Figs. 1A; 2A, C) are distinguishable in life behind the base

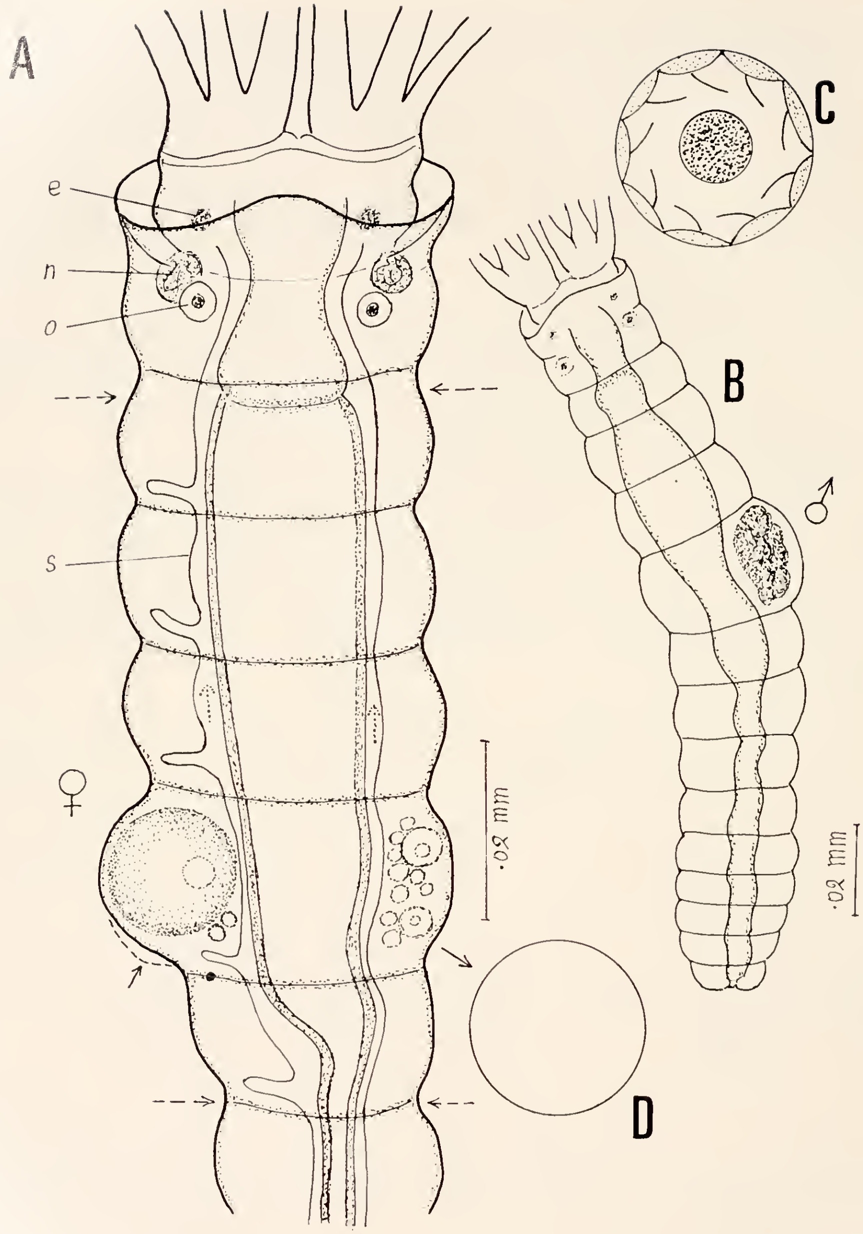

FIGURE 2. Male and female individuals seen from ventral side (not to same scale), parapodial structure not shown. A illustrates the thorax and head region of a mature female, the head showing prostomial ocelli, collar, and "prethoracic" setigerous segment with otocysts and nephridia; the junction between the oesophagus and thoracic stomach is indicated by the distal broken arrows, the junction between thoracic gut and abdominal intestine by the

of the collar, in the first setigerous segment. Each otocyst consists of a closed vesicle containing a birefringent granule and is formed by flattened epithelial cells, about eight in optical section, each bearing a long cilium or flagellum. The granule exhibits a barely discernible movement.

Internal organization and activity

A pair of nephridia, located very close to the otocysts, brownish in life, open anteriorly into the trough formed by the collar with the prostomial crown (Fig. 2A). In most sabellids this pair of nephridia extends posteriorly throughout the thoracic segments. Here, they remain restricted to the first such segment, as in serpulid polychaetes.

The form and subdivisions of the gut are readily seen in the living state. Three regions are clearly distinguishable, namely, prothoracic, thoracic and abdominal (Fig. 2A). The prothoracic, or oesophageal region extends from the mouth to the posterior limit of the first setigerous segment. At the junction with the thoracic gut, a dark narrow transverse heavily-ciliated band indicates a sharp transition from the light brown thin-walled oesophagus to the dark brown thicker wall of the thoracic region or stomach (Fig. 2A). The stomach extends posteriorly to the limit of the thoracic segments, *i.e.*, to the thorax-abdominal junction, indicated externally by the parapodial dorso-ventral inversion, where it changes sharply to a relatively narrow intestine extending to the pygidium. This conformity of internal, gut differentiation with external, body wall differentiation holds for both juvenile individuals which have four fully thoracic segments and for mature individuals which have five (segments with both setae and hooks, and excluding the first setigerous segment which has setae but no hooks).

The conversion of a four-thoracic segment juvenile to a five-thoracic segment adult involves the external body wall (epidermis) and the gut to the same extent. One complete segment transforms from abdominal to thoracic type (Fig. 1A, E), with dorsal setae and ventral hooks replacing dorsal hooks and ventral setae.

Most of the gut is enclosed by a vascular sinus containing greenish (choloro-cruorin) blood (Fig. 2A). Regular peristaltic waves in the sinus wall commence at the posterior end, in the first prepygidial segment, and move anteriorly to the junction of stomach and oesophagus, where they cease. Blood flows steadily and continuously forward through the sinus to the prothoracic nephridia and the peristomial structures, but is not actively propelled by the prothoracic segments. There are no branchial hearts. Notably, in the thoracic segments proper, but not in the abdomen, a blind lateral diverticulum extends from the sinus in each segment, on the right side only (Fig. 2A). These almost certainly correspond to the paired lateral blind vessels typical of other sabellid polychaetes. It is of interest that when the first abdominal segment transforms to bcome the fifth thoracic segment, a typical diverticulum develops in that segment (Fig. 2A).

lower broken arrows. The whole thoracic gut is enveloped by a vascular sinus with blind lateral branches on the right side (left side of drawing), the dotted arrows showing direction of waves. Oocytes form only in the fourth thoracic segment and mature singly, alternatingly one on each side. B shows mature male with testis on right side only; C, enlarged drawing of otocyst; and D, ripe egg at same scale as A, immediately after being extruded from the fourth thoracic segment.

Individuals become sexually mature following the conversion of the first abdominal segment to become the fifth thoracic. Gonads develop only in the fourth thoracic segment (Figs. 1A, 2A). Sexes are separate. An ovary develops on each side when the abdominal region of the worm consists of about seven segments, the usual maximum number in females. The mature ovum is approximately 140 microns in diameter, which may be more than half the entire width of the worm. Only one ovum at a time grows to maturity, apparently alternating in one side and the other. It escapes by rupture (possibly pore expansion) of the posterolateral segmental body wall of the respective side (Fig. 2A). The next largest oocyte is seen on the opposite side. Altogether as many as nine oocytes have been seen in squashed individuals. Simultaneous full growth of more than one oocyte would inevitably be too destructive to adjoining structures and probably to the whole organism. In male individuals a testis develops only on the right side of the fourth thoracic segment (Fig. 2B), in individuals with at least seven abdominal segments. As many as nine abdominal segments have been seen in male individuals.

Discussion

The marine interstitial habitat is typical for newly settled larvae of diverse kinds, usually following larval metamorphosis. The survival of such minute organisms in an environment of unstable sand grains or detritus is commonly an essential prelude to further growth and subsequent reproduction. Whether the final state is mobile or sedentary, and however large or small, an initial explorative phase is common during postlarval development. Postlarval organisms may accordingly make this their permanent way of life on two conditions, namely, that they do not grow beyond a critical size relative to that of the environmental particles and that they attain sexual maturity. The species described here meets both conditions.

To begin with, juvenile features are retained into the adult state. This is most evident in the number of thoracic and abdominal segments produced, in the number and nature of the tentacles comprising the prostomial crown, and in the abortive and one-sided development of the segmental blood vessels. Thoracic segments are initially four and through abdominal transformation increase to five, whereas the number of thoracic segments typical of the family is seven or eight, irrespective of the number originally present in the postlarval stage. Tentacular radioles are restricted to five on each side, two of which remain small and without filaments, which is a transient phase of growth for members of all larger species. Gonads, male or female, respectively, develop only in a single specific throacic segment. Prepygidial addition of new abdominal segments merely increases the number from the five originally present in the newly settled individual to less than twice that number. Everything indicates a cessation of growth at the equivalent of a very early juvenile stage.

The growth and release of a single egg, one at a time, is obviously the absolute minimum for sexual reproduction, and is an invariable characterstic of this species. Two questions arise. Could smaller eggs, permitting smaller body sizes for sexually mature individuals, serve the developmental or adaptive needs of the species? The answer is conditional. The eggs of many polychaetes are small, with little yolk, with a diameter within 60 to 80 microns (Marsden, 1960; Shearer, 1911). Such eggs develop typically with trochophore larvae, with a feeding planktonic phase before settling. This is true of those species of serpulids so far known, a group which is united with sabellids to comprise the Sabelliformia. Conceivably, therefore, sabellids could persist even if their eggs were of the same small dimensions. Eggs of sabellids, however, are, without exception, so far as is known, considerably more yolky and have a diameter ranging from about 140 to more than 200 microns, which not only results in a comparatively larger size at the time of settling but also obviates both the need and even the capacity to feed before doing so. This interstitial species, therefore, can be said to represent the extreme condition of dwarfism possible for a sabellid species without involving a radical change in developmental procedure.

Gonads first appear and grow only when body growth as a whole is complete. Growth of the one seems incompatible with that of the other, which could mean merely that, once gonad development has been initiated, virtually all nutrition is diverted to gonadal growth. Whatever truth may lie in this explanation, however, it is not the whole answer. Gonads may form because growth generally has ceased, or general growth may cease because gonads are differentiating but not necessarily as the result of nutritional competition (Berrill, 1961). In any case a question remains. Why do new segments fail to appear in the prepygidial growth zone even in their initial minute form, or, alternatively, why is sexual maturity so precocious? In relation to the latter, what little is known concerning polychaete neurohormones indicates that their effect is to inhibit, rather than stimulate, the onset of sexual maturity (Golding, 1968). The major problems therefore remain, namely, what determines the limits of organismal growth and in what way does the attainment of sexual maturity relate to this? An entirely comparable phenomenon is seen in ascidians, where interstitial polycarpid and molgulid species of this typically sessile group have become minute and even motile (Monniot, 1965), and are sexually mature when about two millimeters long, with gonads producing only a few eggs only although these are of normal size.

SUMMARY

The anatomy and behavior of a species of the sabellid polychaete subfamily Fabricinae, is described. Mature individuals are virtually microscopic and represent the extreme degree of dwarfism apparently possible for a sabellid. Eggs are matured and liberated unilaterally, one at a time, from a single thoracic segment. Dwarfism in this species is essentially a cessation of body growth at a juvenile stage, accompanied by precocious sexual maturity, as an adaptation to persistent occupation of an interstitial detrital habitat.

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BIOMECHANICS OF WATER-PUMPING BY *CHAETOPTERUS VARIOPEDATUS* RENIER: KINETICS AND HYDRODYNAMICS

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Although a number of previous studies have contributed to an understanding of the mechanism by which *Chaetopterus variopedatus* pumps water through its tube (Joyeux-Laffuie, 1890; Enders, 1909; Berrill, 1927; MacGinitie, 1939; Wells, and Dales, 1951; Dales, 1969; Aksyuk and Sveshnikov, 1971; Brown, 1975), the functional design and operation of the integrated three-segment pump system have not been examined in detail. In addition, there has been virtually no systematic attempt to gather data which would permit a quantitative assessment of the pumping performance to be made. This paper presents an operational analysis of the pumping mechanism of *Chaetopterus* which draws on the approaches employed in mechanical and hydraulics engineering.

MATERIALS AND METHODS

Source and maintenance of animals

Specimens of *Chaetopterus* were obtained from Pacific Bio-Marine Laboratories, Inc., Venice, California. Aquarium maintenance procedures and the details of the artificial glass tube-houses are given in earlier papers (Brown, Bdzil and Frisch, 1972; Brown, 1975; Brown and Rosen, 1977).

Mechanical events

In order to correlate the mechanical events of the pumping cycle with the fluctuations in water flow through the tube-house, an apparatus was developed which permitted recording of the top and side views of an actively pumping animal and, simultaneously, the static discharge pressure. The viewing chamber, reservoirs, and photographic set-up have been previously described in detail (Brown, 1975). For pressure measurements, a machined Lucite coupling of the same internal diameter as the glass house was attached to one end of the tube. A static pressure opening, perpendicular to the water flow and 1 cm from the end of the tube, led directly to a Statham UGP-5 low-pressure diaphragm and Universal transducer. Signals from the preamplifier/meter were fed into a Texas Instruments Servo/Riter (0.1 mV full-scale) amplifier/linear recorder. A light bamboo pushrod was attached to the pen of the recorder and positioned so that a paper indicator arrow at the end of the pushrod was within the field of view of the camera. Calibration of the pressure transducer was done before and after each period of filming by lowering the water level in the reservoirs by 1.0 mm and observing the recorder response. Correlation of activity with pressure (and hence flow) changes was accomplished by comparing data from frame-by-frame analysis of films with the records from the strip-chart recorder.

Flow rate

The rate of water flow through the tube was measured by means of a bubble flow-meter. This design has the combined advantages of generating virtually no back-pressure as well as measuring the velocity of the entire water column, rather than just certain parts. For this purpose, a 15 cm glass tube of internal cross-sectional area slightly greater than that of the terminal tube constriction was interposed between the Lucite coupling and the reservoir. Indicator marks 10 cm apart were scribed on the tube, and the volume of the tube between the marks precisely determined. An air bubble could be injected *via* hypodermic syringe and flexible tubing through an injection port in the coupling. For any single determination, a series of ten bubbles was injected into the discharge current and the elapsed time between the marks determined by electronic stop-watch for each bubble. A mean transit time and, ultimately, an average volume flow rate could then be calculated. Since simultaneous recording of pressure changes also gave the stroke rate, the mean stroke volume could readily be determined.

Oxygen consumption

The concentrations of oxygen in the water at the inflow and discharge ends of the tube were determined by means of a YSI model 57 oxygen meter. Water samples were pumped by means of a Cole Parmer peristaltic pump through a flow chamber (volume = 0.7 ml) past the electrode membrane at a rate of 1.0 ml/min until a stable reading was obtained. Appropriate corrections for atmospheric pressure, salinity, and temperature were made. The oxygen consumption of an actively pumping worm was determined by taking the difference in oxygen concentrations between inlet (mean of two determinations) and discharge ends of the tube and multiplying by the mean volume flow rate. Measurements of oxygen consumption by inactive worms were done according to the procedure of Dales (1969).

Performance measurements

In determining the performance characteristics of an individual worm, the following procedure was followed: (1) acclimatize worm in a glass tube-house for at least 1 week; (2) transfer tube to experimental set-up for at least 12 hrs; (3) measure and record atmospheric pressure and water temperature; (4) determine oxygen concentration of incurrent water supply; (5) calibrate pressure transducer; (6) start pressure/rate recorder; (7) when stroke rate steady, measure volume flow rate; (8) determine oxygen concentration of discharge current; (9) remeasure volume flow rate; (10) remeasure oxygen concentration of incurrent water supply; (11) recalibrate pressure transducer. At this point the pressure/rate recording would be examined to ascertain if constant performance (as judged by stroke rate and pressure output) had been maintained throughout the sampling interval. If within the sampling period the stroke rate varied by more than 2 strokes/min or the peak discharge pressures varied by more than ± 0.2 mm

H_2O, the entire procedure was repeated. If the performance of the worm was consistent within these limits, the procedure was continued as follows: (12) remove worm from tube, blot and determine wet weight; (13) transfer worm to sea water containing 0.075% w./v. tricaine methansulfonate ("MS-222", Sigma); and finally (14) when the worm was immobile, determine O_2 consumption (standard metabolic rate). The total procedure, although laborious, provides direct measurement or permits computation of nearly all relevant performance characteristics for each experimental animal.

RESULTS

The tube-house as a water conducting cylinder

Since the geometry and dimensions of the conduit are of great importance in determining the overall performance characteristics of any liquid pumping system, these parameters will be considered first. The tube-house of *Chaetopterus* is equivalent, in the present context, to a pump piston-chamber plus all of the pipes and "fittings." It is constructed of layers of protein fibers embedded within an acid mucopolysaccharide matrix (Zola, 1967; Brown and McGee-Russell, 1971). This composite material renders the tube pliable but inelastic for all practical purposes. Enders (1906) has given a general description of the methods by which a worm builds and modifies its tube, and two features of the construction process are especially pertinent for the present consideration. First, new layers of protein/polysaccharide are continuously applied to the inner surface of the tube by the worm. This results in the inner surface being always smooth and somewhat slick or slimy (presumably due to the mucopolysaccharide component). Secondly, during its lifetime a worm periodically enlarges its tube to accommodate its increasing bulk. These periodic renovations are carried out upon relatively short segments of the tube at any one time and are influenced by the physical surroundings. Therefore, the shape and size of a tube varies from one worm to another, as well as from one time to another for any individual worm.

In an attempt to determine whether there exist any constant features of tube design, twenty-five recently collected tubes and their living worm occupants were examined and measured. The tubes were selected to include as wide a size range as possible, and to include a variety of shapes—from nearly straight to markedly contorted. The tubes were measured as follows: total length, internal diameter at 1-cm intervals, and minimum internal diameter of the terminal openings. For the worms, wet weight, diameter at segment 12, and total length (extended and contracted) were measured. Such data led to the following conclusions: the length of the tube is poorly correlated with the "size" (any measure) of the worm inhabitant, with the exception that the tubes were always longer than the extended worm; the average internal diameter of main body of the tube was closely correlated with the "size" (especially diameter) of the worm, and hence is considered to be the best measure of the "size" of the tube; even in the most contorted tubes, the variation in internal diameter of the main part of the tube was small—being less than ± 10% of the mean internal diameter; and the terminal openings of the tubes were invariably constricted—the mean ratio of orifice cross-sectional area to mean tube cross-sectional area was 0.190 ± 0.080 (s.d.).

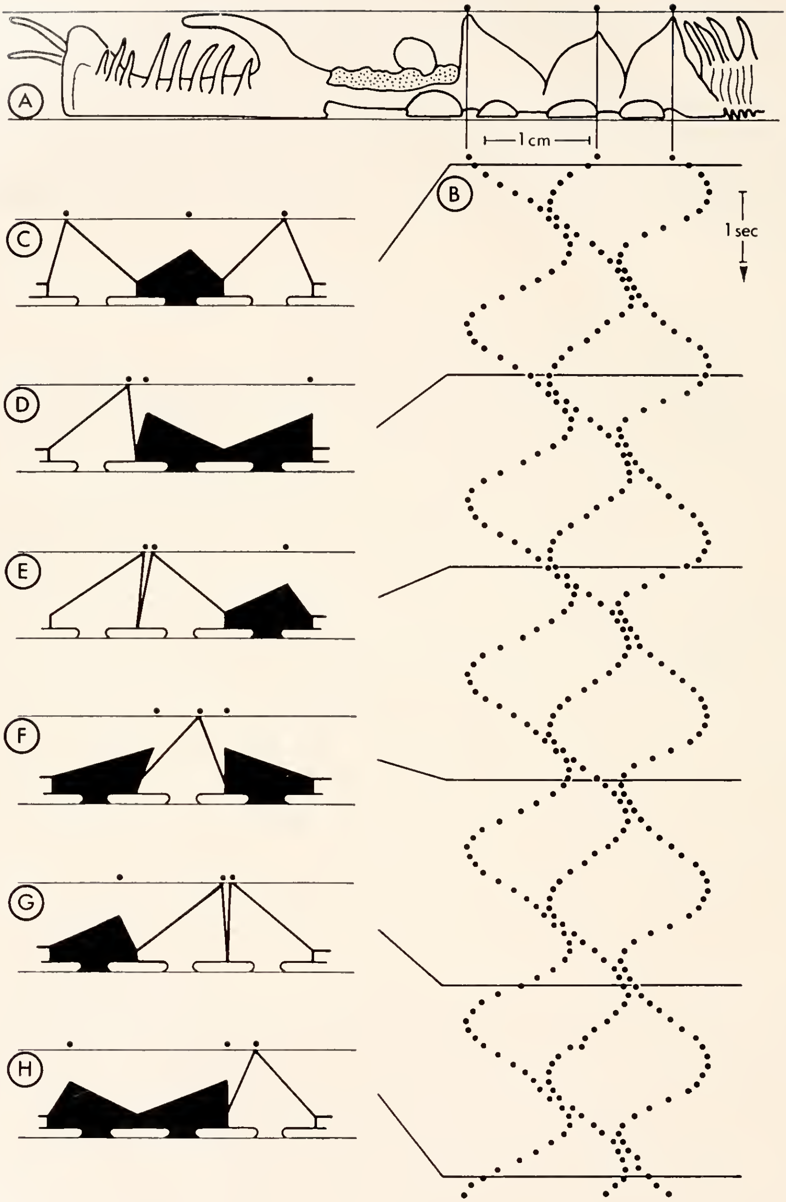

Figure 1. A, Orientation and posture of *Chaetopterus* during water-pumping (measurements taken at reference points indicated); B, frame-by-frame analysis of displacements of piston segments during six consecutive strokes; C-H, diagrammatic representation of position and shape of piston segments at the times indicated (white = power phase; black = recovery phase).

Qualitative performance

The moving parts of a commercial reciprocating pump consist, at a minimum, of power train, pistons, and valves (Hicks, 1957). The analogous elements are represented in segments 14–16 of *Chaetopterus* by the intrinsic musculature (largely the promotor and remotor muscles), the posterior segmental wall, and the parapodial margins, respectively (Brown, 1975). Figure 1, A-H, illustrates the basic working features of the integrated 3-piston pump mechanism of *Chaetopterus.* Noteworthy are the virtually identical repetition of the motions (axial displacements illustrated) which occur during the six successive stroke cycles presented here, as well as the high degree of precision in the coordination of the pumping and valve movements, both within each individual segment and between the three adjacent segments (see also, Brown, 1975, Fig. 8). It is evident that during normal pumping the waves of activity are retrograde; that is, they begin anteriorly at segment 14 and move posteriorly to segment 16. The phase difference between piston units would be predicted to be 1/3, and this is found to be the case observationally. The smooth transitions observed between segment 16 (most posterior) and segment 14 (most anterior) argue for a high degree of intersegmental coordination during continuous pumping activities, and this is also suggested by the work of Berrill (1927). Comparison of simultaneous recordings of the piston movements with fluid discharge pressure (Fig. 2) gives a clearer picture of the detailed operation of the moving elements of the system. It can be seen that the discharge pressure (and hence water flow) is pulsatile, with output maxima corresponding to the periods of maximum sustained rearward velocity of the piston segments, and output minima occurring during the overlap and changeover between the different piston/valve units. As before, the coordination of events during the changeover between segments 16 to 14 is indistinguishable from those of the structurally-adjacent segments (*i.e.*, 14 to 15 and 15 to 16). The data clearly show that at least one piston is in its power phase at all times, and also that at least one set of valves is in its closed configuration at all times. These latter two features have great functional significance, since they ensure that fluid flow is continuously maintained throughout periods of active pumping.

With regard to the direction of flow, it is my experience that a worm does not reverse the direction of the effective piston stroke during normal water pumping activities. Rather, a worm turns end-for-end in its tube in order to reverse the direction of flow in the system. Thus, although the functional inlet and discharge ends of the pump system are reversed, the water flow across the worm remains directed anterior to posterior. This constancy of anterior-to-posterior water flow is clearly dictated by the linearly polarized structures associated with the unique mucous-bag feeding mechanism of the animal (MacGinitie, 1939). That worms may, exceptionally, reverse the direction of beating has been previously reported by Enders (1909) and Werner (1959), but it is likely that such reversals were associated with tube-cleaning activities, which are produced by a motor sequence quite different from that normally employed (Brown and Rosen, 1977). As stated earlier (Brown, 1975), *Chaetopterus* shows no evidence of preferring one end of its tube over the other insofar as direction of water flow is concerned. In contrast, there are clear preferences in body orientation (ventral side down) and position (closer to the incurrent opening). These latter

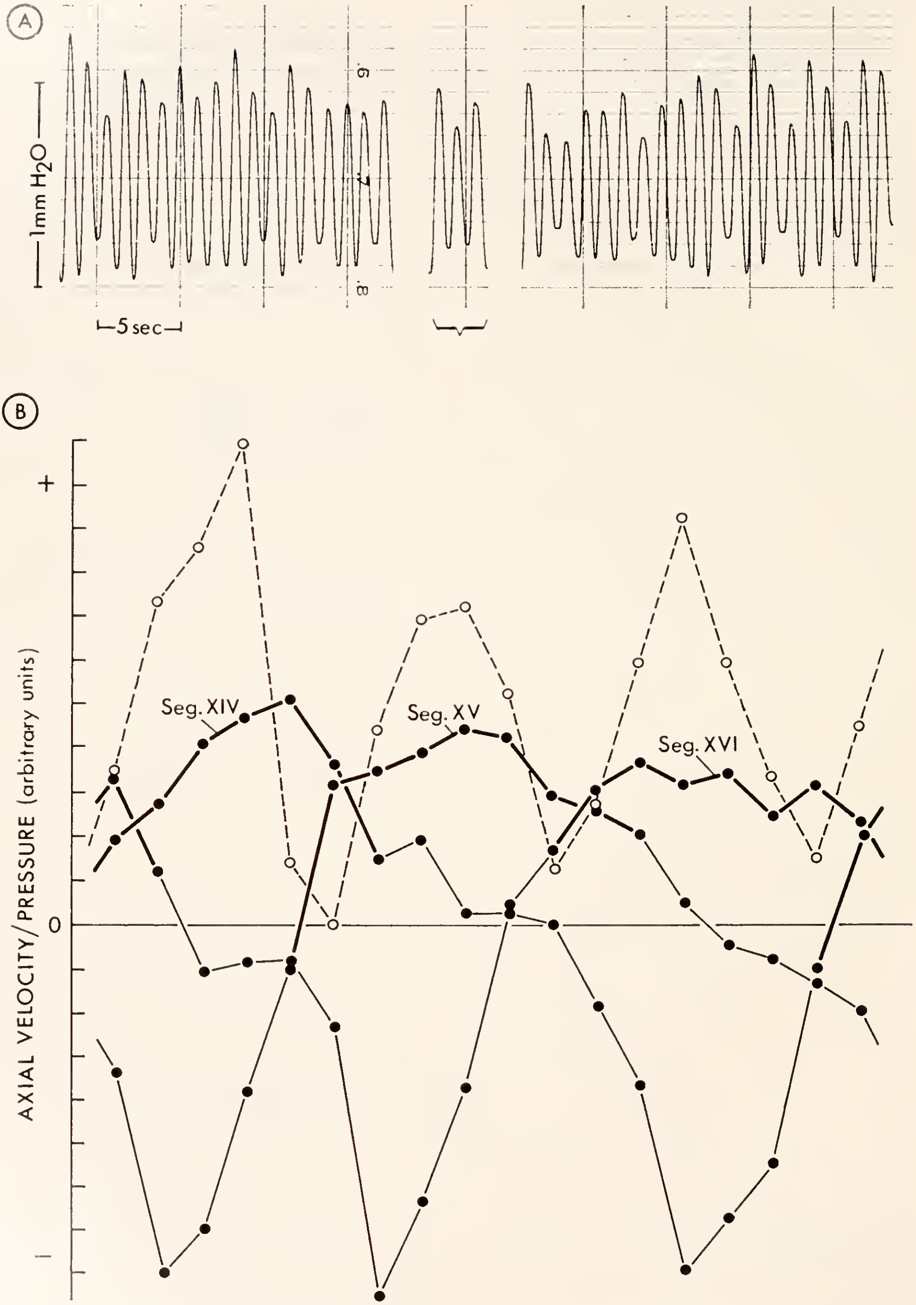

FIGURE 2. A, Strip-chart recording of static discharge pressure during water-pumping; and B, frame-by-frame analysis of the one complete stroke cycle indicated (dashed line shows

characteristics, however, appear not to be critical factors in the coordinated operation of the pump mechanism.

Quantitative performance

It is readily observable that an individual animal has considerable latitude in the rate of pumping which it can accomplish. In addition, the extensibility of the three pump segments permits a worm to achieve variable displacement during the power stroke. These two capabilities clearly enable worms to make volume-flow adjustments by changing either stroke rate or stroke volume, or by a combination of the two. The particular circumstances under which a worm would choose one method over another are quite unknown. In fact, even the relationship between pumping rate and such obvious external conditions as temperature, dissolved oxygen or carbon dioxide, and/or the presence of nutrients has not been examined systematically. The previously published data on the quantitative aspects of water-pumping by *Chaetopterus,* along with results from the present experiments, are summarized in Table I. With the exception of the flow rate estimate of Aksyuk and Sveshnikov (1971), the data appear reasonably consistent, given the small number of animals which have been measured and the many unknown factors which may influence performance. The measured and calculated values of Wells and Dales (1951), Dales (1969) and the present paper are at least of the same order of magnitude.

DISCUSSION

Notwithstanding the conventional description of *Chaetopterus* tube-houses as being "U-shaped", it is evident that such tubes may be constructed in a variety of forms. Such differences in overall shape undoubtedly reflect the environmental peculiarities of the site where each tube-house was constructed (Enders, 1906; 1909). However, since all of the houses examined were obviously functional, it appears unlikely that the gross shape of the tube is of critical importance in the water-pumping mechanism. In contrast, certain other features of tube-house design can be shown to bear directly on the water conducting function. For example, the tubes of *Chaetopterus* are unique among those of the Chaetopteridae in possessing identical terminal openings of relatively large cross-sectional area (for descriptions of the tubes of *Spiochaetopterus, Telepsavus, Phyllochaetopterus, Ranzanides,* and *Mesochaetopterus,* see Barnes, 1964; 1965). Such apertures are an obvious requirement if any large volume of water is to be transported through the tube. The nonpolarity of the tubes, together with the ability of worms to reverse themselves rapidly, permit either end of a tube-house to serve as a functional intake or discharge opening. This is of considerable importance in maintaining unobstructed water flow during potentially-fouling water conditions (Brown and Rosen, 1977). The terminal constrictions serve several functions. Besides acting to keep larger predators out of the tube, such constrictions serve to increase

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position of pushrod indicator (pressure); solid lines are derived axial velocities of the three piston segments; positive velocity values for power stroke, negative values for recovery stroke; heavy lines indicate lateral margins of segments pressed against inner wall of tube).

Table I

Quantitative performance of Chaetopterus *during water-pumping activity; values in brackets were calculated from data given in the reference cited.*

Parameter (units)	MacGinitie, 1939	Wells and Dales, 1951	Dales, 1969	Aksyuk and Sveshnikov, 1971	This paper, mean (range)
Number of animals	2	—	1	—	5
Wet weight (grams)	—	—	4.32	—	4.52 (3.18–6.40)
Water temperature (°C)	—	11–16	15	24	16
Stroke rate (strokes/sec)	1.07, 1.03	—	—	[0.21–0.97]	0.53 (0.45–0.67)
Stroke volume (cm^3/stroke)	—	0.27	—	[5.2]	1.01 (0.88–1.17)
Flow rate (cm^3/sec)	—	0.27	0.13	*ca.* 5.0	0.54 (0.43–0.70)
O_2 consumption [cm^3/g·hr)]	—	—	0.225	—	0.124 (0.050–0.200)
R.Q.	—	—	1.0	—	—
Discharge pressure (N/m^2)	—	—	[10–20]	—	10–20

the water velocity at the incurrent and discharge openings above that in the main part of the tube. In the present sample of tubes the orifice velocity is about five and one-half times greater, but it is apparently more than twenty-five times greater in the large *Chaetopterus* tubes from the Sea of Japan measured by Aksyuk and Sveshnikov (1971). Although high intake velocity may be important in facilitating capture of actively swimming zoo-plankton, as suggested by Aksyuk and Sveshnikov (1971), a high discharge velocity is probably equally important in serving to eject feces and other potentially-fouling particles to a considerable distance from the tube (*cf.*, analogous situations in sponges, acsidians, etc.). In addition, it is likely that such high water velocities serve to prevent sessile organisms from settling on the orifice rims and subsequently obstructing a free flow of water.

The relative invariance in the internal diameter of a tube-house reflects the manner in which it is constructed—*i.e.,* as layers secreted from the convex "ventral shield" located on the anterior 12 segments of the worm (Brown, unpublished observations). This secretory "shield" also acts as a template upon which the protein/polysaccharide layers assume their final (curved) shape. The isodiametric nature of the major portion of the tube is functionally significant in that it permits a worm to pump water from virtually any location within its tube with equal (and presumably optimal) effectiveness. In addition, the slick/slimy nature of the newly-secreted wall of the tube acts, in concert with epithelial mucins secreted from segments 14–16, as a lubricant to minimize friction between the moving piston segments and the wall of the tube.

In operation, *Chaetopterus* is most closely analogous to a reciprocating positive-displacement pump (Wilson, 1950; Brown, 1975). The pumping system of *Chaetopterus* can be further categorized as being "compound" (since more than one piston/valve unit is involved) and "single-acting" (since liquid is pumped only during half of the stroke cycle) (Hicks, 1957). In a simple single-acting power reciprocating pump, the discharge curve (showing output pressure or flow) invariably takes the form of rectified sine wave, with flow pulses (lasting for one-half the complete stroke cycle) alternating regularly with periods of zero flow. The addition of other single-acting piston/valve units with overlapping power strokes has the dual advantages of reducing the magnitude of pulsations as well as assuring that flow will be continuous. The multiple piston/valve units of *Chaetopterus,* although differing from commercial pumps by being arranged in series rather than in parallel, achieve precisely the same effects—namely, dampened pulsations and continuous flow. In *Chaetopterus,* pulse dampening is undoubtedly augmented by the deformability of the "pistons", as well as the smooth integration of motions during periods of transition from one pump segment to another (Fig. 2B). It is of special interest to note that in compound single-acting power reciprocating pumps the *minimum* number of piston/valve units which can yield a continuous flow is three (Hicks, 1957). The fact that *Chaetopterus* possesses just this number of pump segments may indicate the existence of a strong selective advantage in being able to maintain a continuous water flow during periods of active pumping. It may be further mentioned that *Chaetopterus* compares very favorably with the better mechanical pumps (Hicks, 1957; Wilson, 1950) in having the ability, during "operation", to vary the stroke rate, vary the stroke volume, and reverse the direction of flow. Its self-cleaning (Brown and Rosen, 1977) and self-repair (Enders, 1906) capabilities are "features" unrivalled in mechanical systems.

From the present data, quantitative estimates of power output, power input, and aerobic mechanical efficiency can be made. Power output is obtained from the volume flow rate multiplied by the total pressure drop across the "pump" (see Alexander, 1975, pp. 28–30, for a well-illustrated discussion). In the case where the mucous feeding bag is not deployed (the situation which prevailed during all of the present experiments), the discharge pressure varied in a pulsatile fashion between 1–2 mm H_2O (= 10–20 N/m^2). Although the pressure on the intake side of the piston segments was not measured simultaneously, it may be assumed to be of equal magnitude. Taking 15 N/m^2 to be a reasonable average value for both the intake and the discharge pressures, the sum total pressure drop across the pump segments is on the order of 30 N/m^2. Taking 0.5 cm^3/sec as the mean flow rate, the power ouput is calculated to be 1.5×10^{-5} Joules/sec. At the same time, the total oxygen consumption due to pumping activity, per worm, was measured to be *ca.* 0.5 cm/hr^3. Employing Dales' (1969) measured R.Q. of 1.0 (and therefore an equivalent of 5.09 calories of energy liberated per cm^3 of O_2 consumed), the equivalent aerobic power input is calculated to be 3×10^{-3} Joules/sec. The overall water-pumping efficiency of a nonfeeding *Chaetopterus* is thus seen to be about 0.5%. In the alternative case, *i.e.,* during periods when the mucous bag is stretched across the lumen of the tube, it is possible that the power output is somewhat greater than the above estimate would suggest. For this to be true, the pressure differential across the mucous sheet would have to exceed 3 mm of H_2O, with no diminution in flow rate. Although it is theoretically possible to determine this value directly (*i.e.,* by measuring the pressure at a point between the mucous sheet and the first pump segment), numerous attempts to do so have thus far proved unsuccessful. Moreover, indirect calculation of the total porosity of the mucous sheet is not feasible at present, in spite of the availability of data on the effective size of the individual pores (MacGinitie, 1945; but see Jørgensen, 1966, for a critical discussion). However, inasmuch as there are no discernable differences in the pumping behavior of worms with, or without, a mucous sheet present (indicative, for example, of different "loads" on the pump mechanism), and the area of the mucous bag is nearly 8 × greater than the cross-sectional area of the tube (calculated from MacGinitie, 1939), it is tentatively concluded that the power developed by a worm, while feeding, is not likely to be significantly different from the value reported here.

As a final point, an assessment of the nature of the "work" performed by the pump mechanism of *Chaetopterus* may be attempted. Reference to analyses of comparable mechanical pumping systems (Hicks, 1957; Wilson, 1950) provides an approach to the problem. The total work performed by most reciprocating liquid pumps is the sum of three identifiable components: first, the work done to lift a mass of fluid to a higher level in the gravitational field; secondly, the work done to overcome resistance to flow (= friction) caused by the inner surface of the piping and various "fittings" (elbows, valves, constrictors, etc.); and thirdly, the work done (at each power stroke) to accelerate a mass of fluid to a greater velocity (= inertial resistance). In determining the magnitude of the lift component, reference must be made to the height differential between the liquid surfaces at the supply and delivery ends of the pump. For *Chaetopterus,* it is evident that this difference is zero, since the level(s) of the ocean's surface above the intake and discharge ends of the pumping segments are effectively identical at any given instant. *Chaetopterus,* therefore, does no lifting work, and thus corresponds to the so-called "transfer pumps" used widely in industry to move liquids horizontally from one container to another. Given a liquid pumping system in which the work done is limited to that overcoming frictional and inertial resistances, procedures developed in the field of haemodynamics for the analysis of pulsatile flow in rigid-walled ducts (Lightfoot, 1974, pp. 104–113) may be employed. Computation of α^2 (a characteristic frequency-dependent Reynolds' number) from the formula $\alpha^2 = R^2 \omega/v$ (where R = internal radius of tube; ω = stroke frequency, and v = kinematic viscosity) leads to the conclusion that 95% of the work accomplished by *Chaetopterus* during pumping is devoted to overcoming intertial resistance.

The only comparable performance estimates of water-pumping by a worm are those of Chapman (1968) for the echiuroid, *Urechis caupo.* In this animal (which propels water through its burrow by means of peristaltic contractions of the body wall) the calculated power output was in the range of $0.65–3.27 \times 10^{-5}$ Joules/sec, with an aerobic mechanical efficiency of approximately 1.4%. These values are remarkably similar to those reported here for *Chaetopterus.* It would be of considerable interest to have quantitative data on the performance of other animal "pumps", particularly of different mechanical design, to compare with those of *Urechis* and *Chaetopterus.*

I wish to acknowledge, with thanks, Professor R. P. Dales for his helpful criticism on a preliminary draft of the manuscript. I also thank Dr. H. L. Frisch for his continued interest and advice during the course of these studies. I am indebted to Dr. Kenneth P. Able and Dr. Charles Edwards for loans of photographic and electronic equipment; Mr. Edward Donnelly for his help in maintaining the animals; and Mr. Ryland Loos and Mr. Robert Speck for their artistic and photographic assistance.

SUMMARY

1. The activities of the three water-pumping segments of *Chaetopterus variopedatus* are correlated in detail with the fluctuations in water flow through the tube-house.

2. The qualitative performance and design features of the worm's pumping mechanism are compared to those of mechanical reciprocating pumps.

3. Quantitative data on volume flow rate, stroke rate, discharge pressure, and oxygen consumption yield estimates of power output (1.5×10^{-5} Joules/sec) and aerobic efficiency (0.5%).

4. In its natural habitat, *Chaetopterus* does no lifting work, but rather functions solely as a transfer pump.

5. Calculations show that, of the work accomplished by *Chaetopterus* during pumping, 95% is devoted to overcoming inertial resistance.

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COEXISTENCE IN A HERMIT CRAB SPECIES ENSEMBLE

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Recent studies have established that both competition and predation can be potent ecological forces in determining community diversity. However, the task of assessing the relative importance of these two forces within particular communities has barely begun. Schoener (1974) believes that studies of resource partitioning can further an understanding of the influence of competition on the diversity of ecological communities. He emphasizes that such studies must transcend the mere documentation of differences between species and consider the mechanism of competition and certain relationships between the niche parameters of the interacting species..

To keep the number of relevant niche dimensions manageable, studies of resource partitioning are usually confined to ecologically similar species within the community such as a guild or a species ensemble. A guild (Root, 1967) consists of species with similar resource exploitation patterns regardless of their taxonomic relationships. A species ensemble consists of "local, taxonomically circumscribed species assemblages" (Istock, 1973, p. 535). These categories are not mutually exclusive. For example, the members of the hermit crab species ensemble in this study are all local members of the shell-dwelling guild.

Hermit crab species ensembles are ideally suited to studies of resource exploitation because of their common guild membership. Reese (1969) first suggested the applicability of the guild concept to hermit crabs. Vance (1972) later pointed out that hermit crab guilds are appropriate for testing competition theory because much of current theory is based on the assumption that the competing species are limited by a one-dimensional resource and also because hermit crabs appeared to be primarily shell-limited. Recent studies have provided more evidence of shell-limitation (Kellogg, 1976; Spight, 1977).

The purpose of this study was to determine if partitioning of the shell resource could explain the coexistence of seven species of hermit crabs in Beaufort (N. C.) Harbor.

MATERIALS AND METHODS

Sampling procedure

During August and September, 1970, samples for shell utilization data were obtained by dredging with a 40 cm beam scallop dredge in Bulkhead Channel adjacent to the Duke University Marine Laboratory. Each dredge sample was sorted immediately after collection and each hermit crab and empty shell was placed in a compartment of a plastic tackle box. The crabs and shells were transported to the laboratory where they were identified and measured. The sampling area and procedure are described in more detail elsewhere (Kellogg, 1971).

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Shell selection

Subsequent to the previous collections, specimens of *P. longicarpus* and *P. annulipes* were collected from the study area for behavioral experiments. These crabs were held in running seawater tanks, on a natural light schedule, and fed scraps of fish or shrimp. They were exchanged for fresh specimens at weekly intervals.

To determine the preferred shell species, crabs were removed from their shells with an electric soldering gun. The anterior shield length was then measured to the nearest 0.1 mm using the ocular micrometer of a dissecting microscope. Each crab was placed in a bowl containing two shells of different species, both of which were of the preferred size for the crab. Shell width was measured to the nearest 0.1 mm by vernier caliper. The preferred shell width had been determined in previous experiments (Kellogg, 1976). The bowls (250 ml stacking type) contained 1 cm of sand and were placed in a running seawater table. After 24 hours the crab's chosen shell species was recorded. The preference rank order was determined for the four shell species inhabited most frequently by *P. longicarpus* and for the three shell species most frequented by *P. annulipes*. To avoid trying all possible combinations of shell species, it was assumed that if species A were preferred to species B, and B were preferred to C, then species A would also be preferred to species C. This made it possible to determine the preference order of *P. longicarpus* for four shell species with only four shell pairings. A shell preference order of *P. annulipes* for three shell species was determined with two shell pairings (Table III).

This method was chosen in preference to the approach taken by several other investigators (Vance, 1972; Mitchell, 1975; Grant and Ulmer, 1974) to insure that a crab's choice was based on shell morphology rather than on shell size. When species preference is determined by allowing crabs to choose from an assortment of shells similar to that found in the field, some shell species may be unrepresented over a part of their size range. This could induce crabs to select less preferred species (morphologies) to obtain more suitable shell sizes.

Shell fighting

A test for interspecific dominance in shell fighting was conducted using matched pairs of hermit crabs of different species but of the same size. One member of the pair was given a shell of preferred size, the other was given a shell 20% smaller than preferred. Crab size matching was based on the anterior shield length which was measured to the nearest 0.1 mm using the ocular micrometer of a dissecting microscope. Shell size matching was based on shell width (Kellogg, 1976) which was measured to the nearest 0.1 mm with a vernier caliper. Crabs were allowed to enter their assigned shells before placement of the pairs in 20 cm diameter stacking bowls containing 2 cm of sand and 7 cm of sea water. After 24 hours the species inhabiting the shell of preferred size was recorded to determine if a shell swap had occurred. The crab pairs tested were male *P. annulipes*/female *P. longicarpus* and *P. longicarpus*/*P. pollicaris* (both sexes). These pairings were chosen because they represent the groups which overlap the most in their shell size use. Seventeen and twenty trials, respectively, were conducted for each of the species pairs. Approximately equal numbers of trials were begun with each of the species occupying the preferred shell to start.

Habitat selection

P. longicarpus and *P. annulipes* are the most common species in the subtidal regions of the estuaries near Beaufort. *P. longicarpus* occurs on a wide variety of substrates but *P. annulipes* occurs primarily in the shelly sand of the deep tidal channels (personal observation). A substrate selection experiment was conducted to determine if this distribution was due to a preference for a shell substrate by *P. annulipes.* Sand was collected from the intertidal zone near the Duke Marine Laboratory dock, and a 3 cm layer of this sand was placed on the bottom of a running seawater tank measuring 68 cm by 120 cm. Ten to fifteen pieces of broken pelecypod shell freshly collected from Beaufort Harbor Channel were placed in two opposing quadrants of the tank. The other two quadrants were left with just sand. Ten crabs of each species were placed in the tank for each of three trials. The number of crabs of each species found in each quadrant after 24 hours was recorded. The number of crabs in physical contact with the shell material and the number of crabs burrowed in the sand was also recorded.

RESULTS

Shell size exploitation

The shell size niches of the species inhabiting Beaufort Harbor are shown in Figure 1. The crab species are arranged on the abcissa in order of decreasing mean shell size. The major features of this comparison are that both the ratio between the mean sizes of species adjacent on the size scale (r) and the variance in the shell size inhabited by each species increase with increasing mean shell size. These trends are most clearly indicated by *P. annulipes, P. longicarpus,* and *P. pollicaris* which are the most abundant organisms in this collection and are most widely distributed in the Beaufort Harbor area (personal observation). *P. brevidactylus* and *Petrochirus diogenes* are relatively rare inshore and showed relatively minor departures from the values for *P. annulipes* and *P. longicarpus.* Their contribution to the trend might best be described as suggestive.

Shell size overlap between species adjacent on the size scale tends to decrease with increasing shell size (Table I). The highest overlap was for *P. annulipes/P. brevidactylus,* and the lowest was for *P. pollicaris/Petrochirus diogenes.* The overlap between the two most abundant species (*P. annulipes/P. longicarpus*) was also high. Although they are not strictly adjacent on the size scale, this comparison is justified by the rarity of the intervening species (*P. brevidactlylus*). Overlap was measured by

$$D = 1 - \tfrac{1}{2} \sum_{i=1}^{n} |Px,i - Py,i|$$

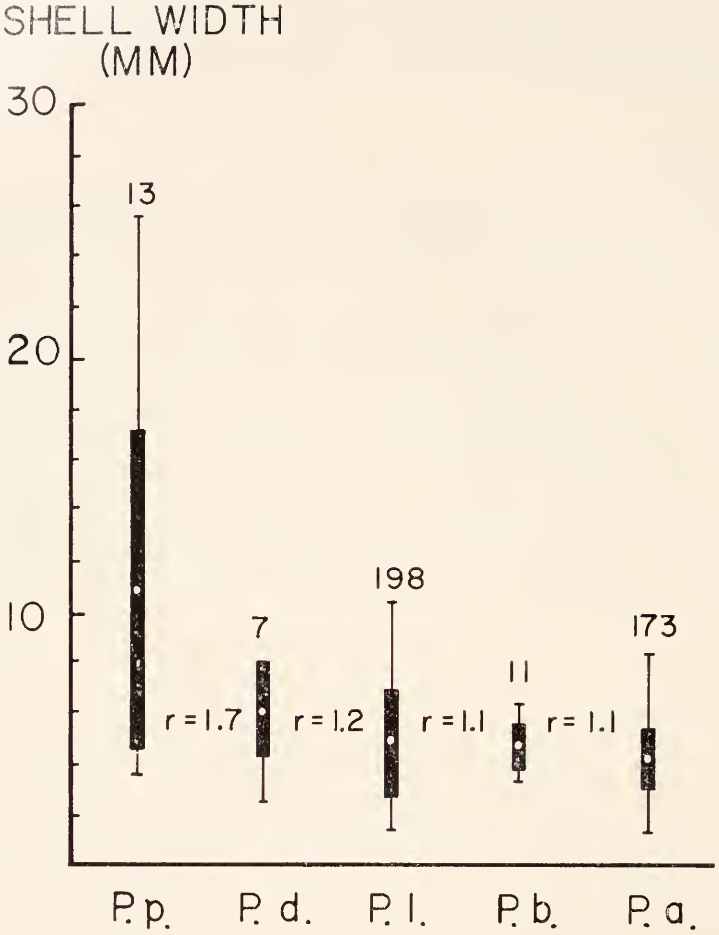

FIGURE 1. Shell size utilization of hermit crabs in Beaufort Harbor. The thin lines show the range, the thick lines show one standard deviation above and below the mean. The ratios of adjacent means (r) are shown between the appropriate species. The number of individuals of each species is given above each line. Species names on the abcissa are abbreviated: P.p., *Pagurus pollicaris;* P.d., *Petrochirus diogenes;* P.l., *Pagurus longicarpus;* P.b., *Pagurus brevidactylus;* and P.a., *Pagurus annulipes.*

where Px,i is the proportion of crab species x inhabiting shell species i and Py,i is the proportion of crab species y that inhabits shell species i. One millimeter size classes were used to calculate overlap.

Shell species exploitation

Each crab species inhabits many species of shells (Table II) but tends to frequent several of them far more than the others. Two shell species comprise more than 50% of the shells utilized by each crab species. By this criterion, the crabs

TABLE I

Species names are abbreviated as in Figure 1 and are ranked in order of increasing mean size. Asterisks indicate species pairs which are adjacent on the size scale.

Species pair	Shell size overlap	Shell species overlap
P.a./P.b.*	0.80	0.74
P.a./P.l.	0.70	0.65
P.a./P.d.	0.37	0.17
P.a./P.p.	0.27	0.30
P.b./P.l.*	0.26	0.47
P.b./P.d.	0.23	0.18
P.b./P.p.	0.32	0.15
P.l./P.d.*	0.39	0.24
P.l./P.p.	0.37	0.33
P.d./P.p.*	0.23	0.66

fall into three shell specialization groups: first, *P. annulipes* and *P. brevidactylus* specialize in *Anachis avara* and *Nassarius vibex;* secondly, *P. longicarpus* specializes in *N. obsoletus* and *N. vibex;* and thirdly, *P. diogenes* and *P. pollicaris* specialize in *Polinices duplicatus* and *Terebra dislocata.*

The differences in shell species inhabitation may be explained partly on the basis of size differences. *P. duplicatus* is one of the few shells large enough for the sexually mature females of the third group. Ovigerous females of the smallest species, *P. annulipes* and *P. brevidactlylus,* are found most frequently in *Anachis avara* shells, the largest of which is too small for reproductively mature individuals of all the other species. Reproductively mature *P. longicarpus* females are found most

TABLE II

Shell species utilized by hermit crabs in Beaufort Harbor. The number of shells inhabited by each crab species is tabulated. Crab species names are abbreviated as in Figure 1.

Shell species	P.a.	P.b.	P.l.	P.d.	P.p.
*Anachis avara**	61	4	16	—	—
A. translirata	6	2	7	—	—
Epitonium sp.	2	—	—	—	—
Eupleura caudata	8	—	8	—	—
Fasciolaria hunteri	2	—	1	—	1
Mitrella lunata	2	—	2	—	—
Nassarius obsoletus	12	—	60	1	1
N. trivittatus	—	—	2	—	—
N. vibex	37	2	57	—	—
Olivella mutica	5	—	5	—	—
Polinices duplicatus	—	—	2	3	6
Terebra dislocata	18	2	18	3	2
T. concava	3	—	—	—	—
Urosalpinx cinerea	12	1	16	—	—
Worm tube	2	—	—	—	—
Other species**	5	—	4	—	3

* See Morris (1973) for gastropod taxonomic authorities.

** Number of species which were represented by only one individual.

frequently in *N. vibex* and *N. obsoletus* shells which are larger than those required by females of the first group and smaller than those required by mature females of third group.

Shell morphology also influences the shell species inhabitation, since differences in shell species inhabitation of *P. longicarpus* and *P. annulipes* exist even within size classes. Shell species overlap within the 1.0–1.4 mm and 1.5–1.9 mm crab size classes is 0.64 and 0.65, respectively. Overlap within the largest size class which contains both *P. longicarpus* and *P. annulipes* (2.0–2.4 mm) is only 0.51. Shell species overlap values were calculated using the formula described above for shell size overlap.

Shell species preference

P. longicarpus, in this experiment, demonstrated a strong shell morphology preference in all of the choices except one (Table III). There was only a weak preference for *N. obsoletus* over *L. irrorata.* The preference order for *P. longicarpus* is therefore *N. vibex* > *N. obsoletus* ⩾ *L. irrorata* > *T. dislocata. P. annulipes,* in this experiment, clearly preferred *A. avara* to *N. vibex* and *N. vibex* to *N. obsoletus.*

Shell fighting

No evidence of interspecific dominance was found among the three most abundant species of this ensemble. No shell swaps occurred in any of the trials, despite the fact that the crabs were allowed to interact for 24 to 48 hours. Possibly a different stress would have elicited fighting. For example, Hazlett (1970) showed that hermit crabs in larger shells rap harder when fighting and this increases their likelihood of evicting an opponent. Therefore, had one crab of the pair been given a preferred shell and the other a shell larger than preferred, the stressed crab might have been more successful. In nature, however, inhabiting a small shell is the more common and more stressful condition for hermit crabs (Vance, 1972; Kellogg, 1976). Interspecific shell fighting is apparently not the cause of the observed shell resource partitioning.

TABLE III

Shell species preference of P. longicarpus *and* P. annulipes. *The probabilities are for the two-tailed binomial test.*

Hermit crab species	Shell species	Numbers chosen	Probability
Pagurus longicarpus	*N. vibex/N. obsoletus*	17/3	0.0026
P. longicarpus	*N. obsoletus/L. irrorata*	14/6	0.1153
P. longicarpus	*N. obsoletus/T. dislocata*	19/1	0.0004
P. longicarpus	*L. irrorata/T. dislocata*	20/0	<0.0001
P. annulipes	*A. avara/N. vibex*	9/1	0.0059
P. annulipes	*N. vibex/N. obsoletus*	10/0	0.0020

Substrate selection

P. annulipes, in this experiment, demonstrated a distinct preference for a shelly substrate by selecting the quadrats having shell fragments more often than those with just sand ($P = 0.004$; two-tailed binomial test). *P. longicarpus* showed no preference for either sandy or shelly substrates. The affinity of *P. annulipes* for shell material was further demonstrated by the observation that 11 of the thirty specimens were in direct contact with shell fragments, compared to only 4 of the thirty *P. longicarpus*. Eleven of the thirty *P. annulipes* were burrowed in the sand at the conclusion of the experiment, as compared to only one *P. longicarpus*. The crabs burrowed in the sand so that their shells were covered but their eyestalks, legs, and antennae protruded. Their antennae were swept forward over the sediment surface periodically in an apparent feeding motion. *P. annulipes* has setose antennae which may trap food particles carried along the sediment surface by tidal currents. *P. longicarpus* lacks such setae and feeds instead by traveling over the substrate and tossing debris into its mouth parts with its minor cheliped (Roberts, 1968). In the estuary, *P. longicarpus* is found on a wide variety of sediments (*e.g.*, sand, mud, shelly sand), but *P. annulipes* is generally restricted to the shelly, sand substrate of the tidal channels (personal observation). The differences in the distribution of these two species are due at least in part to their differing substrate preference and to their differing feeding styles.

DISCUSSION

Schoener (1974) proposes that regular spacing of niches along a single dimension be accepted as evidence of resource partioning generated by competition. He notes that size ratios of adjacent species are typically 1.2 or higher and that in some cases there is a tendency for ratios to increase as sizes increase. The ratios between adjacent species in this ensemble increase from 1.1 to 1.7 as the utilized resource size increases. This indicates that competition and consequent resource partitioning is more pronounced for the larger species. This interpretation is supported by the observation that large empty shells are scarcer than small ones in this area (Kellogg, 1976).

Shell size partitioning seems to be the most important factor permitting coexistence of at least the largest three species in this ensemble, *i.e., Pagurus longicarpus, Pagurus pollicaris,* and *Petrochirus diogenes.* Competition for shells is most severe for these species, and size ratios for adjacent species are in the range reported as the typical degree of size separation for a variety of other organisms in direct competition (Schoener, 1974). Although shell species overlap was also low for these species, this is due mostly to the size differences so shell species partitioning cannot be considered of great importance here. In other locations, many specimens of *Pagurus pollicaris* were collected within the size range of *Pagurus longicarpus,* and their shell species utilizations were similar (personal observation).

The presence of *Petrochirus diogenes* in this ensemble is somewhat puzzling because, although it inhabits shells of relatively large size, no sexually mature individuals were collected either here or in other estuarine collections. Offshore, however, much larger specimens of *Petrochirus diogenes* were collected (even larger than the largest *Pagurus pollicaris*), many of which were sexually mature. This suggests that *Petrochirus diogenes* does not maintain a viable breeding population in this area, but that stray larvae may drift into the estuary from offshore. Some of them apparently locate unused shells which occur in the channels. Shells inhabited by *Petrochirus diogenes* usually were heavily fouled, unlike those found in other areas, and unlike those inhabited by the other species (personal observation). *Petrochirus diogenes* does not prefer shells in this condition. When placed in holding tanks containing a wide variety of shells (conditions, sizes, and species), the *Petrochirus diogenes* specimens readily abandoned their orginal shells for clean ones. The survival of *Petrochirus diogenes* in this area may be attributed to errors of exploitation (Istock, 1973) by *P. longicarpus* and *P. pollicaris* and to partitioning of the shell size spectrum.

Several specimens of two other species, slightly smaller than the mean size of *Petrochirus diogenes,* were also collected in the study area but were not included in the analysis because of insufficient numbers. Both of these species are abundant in certain offshore locations. *Paguristes hummi* was found in *T. dislocata* (the shell least favored by *Pagurus longicarpus*). *Pagurus impressus,* which is similar in morphology to *Pagurus pollicaris* and attains the maximum size of *P. pollicaris* offshore, was collected in this study area, but only as relatively small individuals. *Pagurus impressus* and *Paguristes hummi* can find some small, unexploited shells, but not enough of the appropriate size to sustain a breeding population here.

Shell size partitioning by the smaller species (*Pagurus annulipes, Pagurus brevidactylus,* and *Pagurus longicarpus*) seems less important to their coexistence than for the others. However, it is not negligible. *P. annulipes* and *P. brevidactylus* are very similar in the sizes and species of shells used. They are also very similar in appearance and behavior. There is no apparent difference in their shell use or behavior which can explain their coexistence, assuming that they in fact compete for shells. *P. brevidactylus,* however, is rare in this area but abundant offshore on rocky substrates with a dense fouling community (personal observation). The presence of large pieces of shell covered with a dense fouling community in this area provides an adequate, albeit restricted, substrate for this species in Beaufort Harbor. The existence of *P. brevidactylus* in this area may be due to the presence of shell debris and the availability of a very limited supply of shells not used by *P. annulipes* and *P. longicarpus* (*i.e.*, errors of exploitation). Perhaps the most complex competitive relationship is that between *P. annulipes* and *P. longicarpus.* They are the most abundant species in this study area and are also similar in size and shell species utilization. If *P. brevidactylus* is disregarded because of its relative rarity and *P. annulipes* and *P. longicarpus* are considered as adjacent species on the size scale, we find that they have a size ratio of 1.2, which is within the range suggested by Schoener (1974) as the minimum size separation necessary for the coexistence of competing species along a resource gradient.

In addition to the size differences, shell species utilization differences between *P. annulipes* and *P. longicarpus* are significant. This is shown by the relatively low niche overlap between the two species within the 2.0–2.4 mm size class, which includes mostly reproductively mature individuals of both species.

Schoener (1974) notes that increasing ratios of species sizes are correlated with an increased variance in the food size utilizations of larger species. He states that the variances of food sizes should generally be greater for larger species for two reasons. First, large food items are usually scarcer than small ones and secondly, large species usually have a wider size range. The higher variance, he believes, would cause more overlap (hence more competition) between larger species and would necessitate wider niche spacing.

The trend toward higher variance in shell size utilization for the larger species in this ensemble is accompanied by both of these conditions. First, large shells are relatively scarce (see above) and secondly, the first crab stages of all species are similar so that the size range of species increases in proportion to maximum size.

However, rather than the expected higher shell size overlap with increased crab size, the reverse trend was observed. The greatest overlap was between the smallest species (*P.a./P.b.*), and the least overlap was between the largest species (*P.d./P.p.*). Presumably, overlap among the largest species has been reduced by competitive interactions in the past.

Complementarity of overlap values for two independent niche dimensions can also indicate resource partitioning (Schoener, 1974). For example, hermit crabs with a high size overlap would be expected to overlap less in other dimensions (*e.g.*, shell species) if they are resource limited. Most of the species in this ensemble had an overlap value of less than 0.50 for one or both niche dimensions (shell size and shell species). Only the smaller species (*P.a./P.b.*, *P.b./P.l.*, and *P.a./P.b.*) had high overlap values for both niche dimensions. Furthermore, shell species overlap was greatest between the smaller size classes of *P. annulipes* and *P. longicarpus*. This lack of complementarity among the smaller crabs demonstrates that shell resource partitioning is less crucial to their coexistence than it is for the larger ones.

Shell size partitioning is apparently maintained by differences in growth potential and size at sexual maturity which are evolutionary adaptations to a long history of competition. Partitioning seems not to result from interspecific exclusion by shell fighting, because crabs were not induced to swap shells in laboratory experiments. Nor does it arise from habitat differences in shell size, because both *P. longicarpus* and *P. pollicaris* frequent the same habitats. Furthermore *P. annulipes*, the smallest crab species, does not use large shells such as *Busycon* spp. and *Fasciolaria hunteri*, although these snails inhabit the study area.

Shell species (morphology) partitioning is maintained in part by differences in crab morphology and shell preference. *P. annulipes*, for example, prefers *Anachis avara* shells which are elongate and have a small aperture height relative to the shell width (Kellogg, 1971). Fecund female specimens of *P. annulipes* and *P. brevidactlyus* were most often found in these shells. Female specimens of *P. annulipes* and *P. brevidactlyus* have smaller chelipeds for a given crab weight than males; their chelipeds are also smaller than those of both male and female *P. longicarpus* and *P. pollicaris*. This enables them to use shells with a more restricted aperture, such as *Anachis avara* or *Terebra dislocata*, more effectively. *P. longicarpus*, in contrast, shuns the elongate, heavy, small-apertured *T. dislocata*. Unfortunately, suitable sizes of *T. dislocata* were unavailable when shell preference for *P. annulipes* was determined, but *T. dislocata* would probably rank higher in the preference order of *P. annulipes* than it did for *P. longicarpus*. The suggestion that *P. annulipes* makes more effective use of *T. dislocata* than *P. longicarpus* is supported by the observation that in subsequent sampling offshore, where most of the shells were *T. dislocata,* specimens of *P. annulipes* were predominant.

Habitat differences also help explain shell species partitioning. For example, *N. obsoletus* shells are available to individuals of *P. longicarpus* and *P. pollicaris* because these crabs often forage in the intertidal zone to which *N. obsoletus* is restricted. Individuals of *P. longicarpus* often overturn live *N. obsoletus,* inspect the shell aperture, and then release them, apparently in search of an empty shell or dead snail (personal observation). In contrast, *P. annulipes* individuals were never observed in the intertidal zone. *P. annulipes* is most abundant in the channels along with large numbers of *Anachis avara* which inhabits the fouling community attached to shell debris.

In order of decreasing importance, habitat differences, shell size partitioning, and shell species partitioning maintain the diversity of this species ensemble. Probably only three species (*P. annulipes, P. longicarpus,* and *P. pollicaris*) could coexist in this area if the surrounding areas were homogeneous with the study area, forcing the crabs to rely only on subdivision of the shell resource for ecological differentiation. The presence of the other species in this area is apparently sustained by breeding populations elsewhere, by errors of exploitation of those breeding here, and by the relatively large number of small empty shells.

Among other studies of hermit crab species ensembles, as in this study, it appears that differences in shell size utilization and habitat are most important in allowing coexistence. Grant and Ulmer (1974, p. 40) note that "a large proportion of the *P. acadianus* population exceeds the size range of *P. pubescens*" within their study areas in Maine. They found, however, that the shell species utilization of these crabs was similar within size classes. Vance (1972) has studied shell use in the *P. hirsutiusculus, P. granosimanus, P. beringanus* ensemble in the Pacific intertidal. He found that although *P. hirsutiusculus* preferred shorter, lighter shells than the others, the differences in shell utilization between the three species in the field were slight and not sufficient to explain their coexistence. He did find, however, a marked habitat difference among them which he concluded was the primary reason for their coexistence considering the extensive overlap in shell use. Bollay (1964) studied another group of sympatric hermit crabs of the Pacific intertidal (*P. hirsutiusculus, P. granosimanus,* and *P. samuelis*). She noted that *P. hirsutiusculus* was smaller than the other species and that both *P. hirsutiusculus* and *P. samuelis* attained sexual maturity at a smaller size than did *P. granosimanus.* Furthermore, she found that where *Tegula funebralis,* the largest shell species, was scarce, *P. hirsutiusculus* was relatively abundant, *P. samuelis* was scarce, and *P. granosimanus* was absent. This strongly suggests partitioning of the shell size range although this relationship was not quantified. Samuelson (1970) reports distinct differences in the size range of the four hermit crab species in a subtidal ensemble in Norway. *Anapagurus chiroacanthus* is the smallest of the four, *P. cuanensis* and *P. prideauxii* are intermediate in size, and *P. bernhardus* attains the greatest size. *P. prideauxii* inhabits relatively small shells for its size because of a symbiotic anemone which attaches to its shell. The anemone grows as the crab grows, thus obviating the need to find larger shells. This suggests that shells have been scarce in the past thereby creating evolutionary pressure which caused some species to use other means of protection.

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SUMMARY

The shell exploitation of hermit crabs inhabiting a subtidal region of Beaufort Harbor (N. C.) was studied to determine the importance of shell resource partitioning in allowing the coexistence of the hermit crabs. The three most abundant species (*Pagurus annulipes, P. longicarpus,* and *P. pollicaris*) partition the size range. The size ratio between the larger species (*P. pollicaris* and *P. longicarpus*) was 1.7 and the size ratio between the smaller species (*P. annulipes* and *P. longicarpus*) was only 1.2. Differences in shell species utilization were significant only for the smaller species (*P. annulipes* and *P. longicarpus*). Specimens of four other hermit crab species were collected (*Pagurus brevidactylus, Pagurus impressus, Paguristes hummi,* and *Petrochirus diogenes*). Individuals of these species are more prevalent offshore. In Beaufort Harbor they use relatively small shells or shells which tend to be overlooked or shunned by the other species.

Experiments on shell selection, interspecific fighting, and substrate selection were conducted to determine the mechanism by which habitat differences and shell resource partitioning are maintained. Differences in shell preference and substrate preference were found between *P. longicarpus* and *P. annulipes.* However, no interspecific shell fighting dominance was observed for *P. longicarpus, P. annulipes,* and *P. pollicaris.*

Coexistence of individuals of the seven species of hermit crabs collected in Beaufort Harbor is made possible by a combination of habitat differences, shell size partitioning, and shell species partitioning in descending order of importance. The shell partitioning of the three most abundant species is maintained by differences in maximum crab size at sexual maturity, and by differences in shell species preference. Habitat differences between *P. annulipes* and *P. longicarpus* are maintained in part by a preference for shell debris by *P. annulipes.*

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COCKROACH MOLTING. II. THE NATURE OF REGENERATION-INDUCED DELAY OF MOLTING HORMONE SECRETION

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The study of intrinsic controls of growth and development in organisms has often been aided by the availability of extrinsic cues which permit the organism to be manipulated or stimulated in a close to normal fashion by the experimenter from the periphery. Regeneration of autotomized or lost parts in arthropods is just such a convenient process, often allowing manipulation of the developmental program of the animals. Loss of scales in firebrats (Rohdendorf and Watson, 1969; Larink, 1975) and legs in crustaceans (Holland and Skinner, 1976) result in precocious molting. Excision of wing discs of *Ephestia* (Pohley, 1965) or autotomy of cockroach legs (O'Farrell and Stock, 1953, 1954; Stock and O'Farrell, 1954; Bulliere, 1967, 1968a, b; Kunkel, 1975a) result in a delay of the molting process in order to regenerate the lost limb. In some groups of arthropods there is apparently no modification of the molting cycle to accomodate regeneration (Luscher, 1958; Shaw and Bryant, 1974). Unravelling the mystery of how regeneration modifys the molting cycle in various groups will hopefully result in a deeper appreciation of how the arthropod molting cycle is controlled in general.

Attempts have been made to learn how the endocrine events of the cockroach molting cycle interact with the regenerating limb (Penzlin, 1965). Although progress has been made in understanding the means by which the ecdysone can inhibit and perhaps terminate the molting delay (Bulliere, Buillere and Sengal, 1969; Bulliere and Bulliere, 1970; Marks, 1973; Kunkel, 1975b), there is as yet no concensus as to how the delay of the molting phase is initially established. While earlier experiments suggest that regenerating tissue metabolizes the molting hormone, ecdysone, and as a result prevents molt initiation (O'Farrell, Stock, Rae and Morgen, 1960; Pohley, 1961), the present study reinforces the suggestion (Kunkel, 1975a) that autotomy of a leg inhibits, *via* a nervous feedback, the release of the brain hormone, ecdysiotropin, needed to initiate a molting cycle.

Although previous reports (Brindley, 1897; O'Farrell *et al.*, 1960; Pohley, 1965; Bulliere, 1968a) of positive correlations between amount of amputated or regenerated tissue and amount of molting delay are undoubtedly correct, these correlations, in the case of the cockroach at least, have been misinterpreted. Inferences have been made that the amount of regenerating tissue causes the length of the molting delay. It has been hypothesized (O'Farrell *et al.*, 1960; Pohley, 1965) that the greater the amount of regenerating tissue, the more ecdysone is metabolized and the longer the delay; this will be referred to as the "injury-metabolism model" of molting delay.

Another model, the "nervous-feedback model," proposed in this study, involves a nervous feedback from the ganglion of regenerating limbs to the brain, preventing the release of ecdysiotropin and its subsequent induction of the ecdysone pattern controlling molting.

MATERIALS AND METHODS

Six species of cockroach were used in this study: two from the family Blattellidae, *Blattella germanica* and *Symploce capitata;* two from the family Blattidae, *Blatta orientalis* and *Periplaneta americana;* and two from the family Blaberidae, *Byrsotria fumigata* and *Leucophaea maderae.* Cultures of each species were maintained synchronously at 30° C by controlling their food supply (Kunkel, 1966) and by storing them between molting cycles at 15° C. A strain of *B. germanica* was developed for the purpose of making precise measurements of difference in molting cycle length due to different treatments.

This strain was maintained 50% heterozygous orange body (+/*or*) and 50% homozygous orange body (*or*/*or*) (Ross and Cochran, 1966). The heterozygote and the homozygous recessive larvae were distinctly different in body color and could be separated by superficial inspection. The alleles were found to have no differential effect on the length of the molting cycle. This allowed raising genetically labeled control and experimental groups in the same culture dish, eliminating any between-dish effects on developmental rates.

Autotomy of limbs, nerve section experiments, serum sampling and ecdysterone injection were each performed under brief, light CO_2 anesthesia. Such anesthesia was tested and found to have no measurable effect on the molting cycle length.

A radioimmune assay for ecdysone in insect serum was developed using anti-ecdysone (gift of Dr. John D. O'Connor, University of California, Los Angeles) and ^{3}H-ecdysterone (New England Nuclear, 6 Ci/mmole). The assay was modified from Borst and O'Connor (1974) and Tyler, Hennan, Newton and Collins (1973). Unknown or standard amounts of cold ecdysone were dried down in 0.4 ml polyethylene vials (Thomas) and 6000 dpm or ^{3}H-ecdysone were added in 100 μl of Borate buffer (0.087 N Borate, pH 8.4; 0.075 N NaCl). This solution was mixed to suspend and mix the cold and hot ecdysones. To this mixture 100 μl of 3% anti-ecdysone plus 37% normal rabbit serum in Borate buffer were added and the mixture incubated for 20 minutes at 37° C, followed by chilling for one hour at 4° C. To the tubes 200 μl of saturated ammonium sulfate was added to precipitate γ-globulin and bound ecdysone, the tubes were mixed and after 15 minutes centrifuged in a Beckman microfuge for one minute. The unbound and unprecipitated ^{3}H-ecdysterone in the supernatant was counted: one hundred μl of supernatant + 1.1 ml of water + 10 ml of Triton-X-100 toluene scintillator (Benson, 1966) were counted in a Beckman Scintillation Spectrometer at a tritium counting efficiency of 50%. Control 1 (CPM1) consists of the former complete mixture minus any cold ecdysone to establish the maximum antibody precipitable radioactive ecdysone. Control 2 (CPM2) consists of the complete mixture, minus any cold ecdysone, with the 3% anti-ecdysone replaced with additional amounts of 3% control rabbit serum, to establish the level of nonspecific binding and precipitation. A standard curve of ecdysone bound (CPMX-CPM1)/(CPM2-CPM1) *versus* $\log_2$ cold ecdysone concentration was plotted on probability paper and invariabily gave a straight line. Transformation of percentages to probits allowed linear regression to be performed, and unknown ecdysone values were then computed directly from the regression equation.

Samples for assay of ecdysone were obtained by extraction of 1/20 diluted whole hemolymph with an equal volume of methanol (Bollenbacher, Vedeckis, Gilbert and O'Connor, 1975). The precipitated proteins were removed by centrifugation and the supernatant dried down *in vacuo* at 45° C.

RESULTS

Correlation of degree of injury with delay length

The injury-metabolism model predicts that the length of molting delay is proportional to the size of the eventual regenerate. This correlation can be observed on a number of levels. The most obvious level, in which different anatomical structures are amputated, gives the most dramatic results. Thus the average delay caused by regeneration at 30° C of a femur-tibia-tarsus of fourth instar *B. germanica* larvae is 55 hours, while regeneration of only the tarsus causes a 25 hour delay of molting. On a more subtle level the molting delay associated with regeneration of a particular anatomical structure can vary considerably; the size of the regenerated structure is proportional to the time the structure had available for regeneration. This phenomenon is illustrated in Figure 1. The observed result that delay is a third degree polynomial of regenerate length, suggests that delay is correlated with the volume of tissue regenerated or to be regenerated.

Uncoupling of delay length from regenerate size

To test the causal relaionship between amount of tissue and amount of delay, a series of experiments were designed to see if delay length could be uncoupled from the amount of tissue regenerating. In the first experiment a culture of 240 third instar larvae of the specially developed strain of *B. germanica* were used; 120 heterozygous (+/*or*) larvae had their left metathoracic femur-tibia-tarsus autotomized and 120 homozygous recessive (*or*/*or*) larvae had their right femur-tibia-tarsus autotomized at 40 hours after feeding. This provided after subsequent regeneration and molting two mirror equivalent groups of fourth instar larvae: +/*or* larvae with small left legs but normal right legs and *or*/*or* larvae with normal left legs but small right legs. These fourth instar animals were fed, and 40 hours later either a small or a large metathoracic tarsus was autotomized. The animals were then observed at four hour intervals, and the time of ecdysis and length of the regenerated first tarsal segment was recorded. The sizes of the regenerates of the small *vs.* large tarsal amputees were highly significantly different ($P < 0.0001$) and should have resulted in a substantially different delay if regenerate size was causally related to delay length. However, no such delay difference could be detected between the two regenerate groups ($P > 0.10$). This result suggests that the length of molting delay is associated with some intrinsic property of the thoracic segment and level of autotomy rather than with the size of the amputated part or the size of the regenerate.

A second experiment designed to answer the same question was based on the fact that autotomy of a second leg causes a measurable extra delay when autotomized simultaneously with the first autotomy (Stock and O'Farrell, 1954). In this experiment 832 fourth instar *B. germanica* larvae were split into two groups of 416. At 40 hours after feeding, in one group of 416 either one or two tarsi were autotomized. In the other group of 416 one or two femur tibia-tarsi were autotomized.

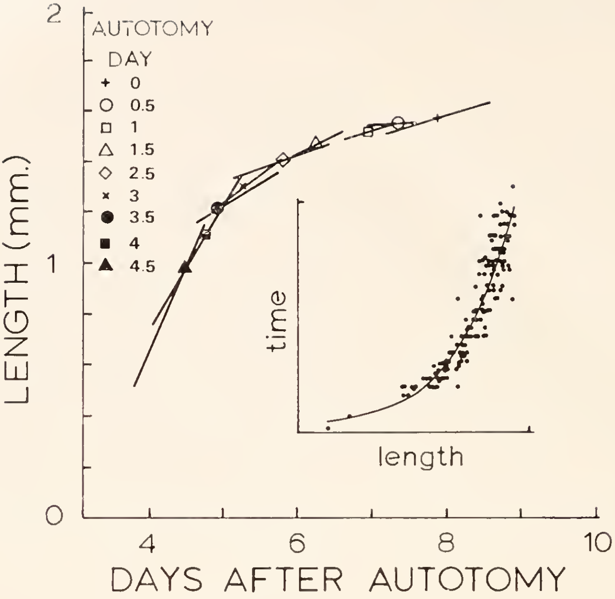

FIGURE 1. Regenerate size as a function of time from autotomy to ecdysis. Each of nine groups of 25 sixth instar *B. germanica* male nymphs had a tarsus autotomized on the specified day after feeding. For each group linear regression was performed on the length of the regenerated first tarsal segment *vs.* the time from autotomy to ecdysis. The regression line for each group is plotted intersecting the mean regenerate size and ecdysis time for that group and spans the range of molting times in its group. Each regression line has a nonzero ($P < 0.05$) positive slope. The inset figure shows a third degree polynomial of length fitted to the times from autotomy to ecdysis.

As in the previous experiment, the time of ecdysis was recorded for each animal in the four treatment groups. Although there was a highly significant 26 hour difference between the delay caused by autotomy at the level of the tarsus versus autotomy of the femur-tibia-tarsus ($P < 0.0001$), and there was a highly significant five hour difference between whether one or two limbs were autotomized in single animals ($P < 0.0001$), there was no significant difference ($P > 0.75$) in the delay caused by a second tarsus as compared to a second femur-tibia-tarsus. Both of these experiments suggest that the bulk of the regenerating tissue has no role in controlling the length of the delay of molting.

Uniformity of delay associated with regeneration

A length-of-delay of molting is associated with each thoracic segment (metathorax > mesothorax > prothorax) and autotomy level (tarsal < femur-tibia-tarsal). This uniform delay for each autotomy type is demonstrated when one measures the synchrony of ecdysis in leg regenerating cochroaches and compares this synchrony to the degree of synchrony achieved simply by controlling feeding. Table I lists for six species of cockroach the mean time of ecdysis and the variance of ecdysis in cultures synchronized by feeding, compared to equivalent

TABLE I

Limb autotomy induced reduction in the variance, $V = (t - \bar{t})^2/n - 1$, *of molting time,* t, *in feeding-synchronized cockroach cultures. Multiple parallel cultures of* Blattella germanica *and* Periplaneta americana *demonstrate the uniformity of variance reduction. Pooled variances for unautotomized (−) and for autotomized (+) cultures were obtained where appropriate prior to calculating the variance ratio, F, testing equality of variance. The probability (P) that such a reduction in variability could have occurred by chance is tabulated. Time is measured in hours from initial food availability.*

Species	Instar	Culture	Autotomy		Culture size, n	Mean molting time, t	V	F (P)
			+/−	time				
Blatella germanica	IV	A	−		132	153	297	2.86 (<0.0001)
		B	−		139	147	184	
		C	−		133	155	248	
		D	−		151	157	260	
		E	−		125	161	243	
		F	−		137	158	159	
		G	−		141	158	264	
		H	−		154	154	181	
		I	+	40	139	212	82	
		J	+	40	121	206	90	
		K	+	40	125	202	59	
		L	+	40	132	198	88	
Periplaneta americana	VII	A	−		61	181	121	1.492 (<0.025)
		B	−		44	215	123	
		C	+	48	51	296	81	
		D	+	48	60	308	82	
Blatta orientalis	V	A	−		292	211	334	1.405 (<0.05)
		B	+	96	71	359	237	
Symploce capitata	V	A	−		165	244	1024	2.32 (<0.0001)
		B	+	96	215	345	441	
Byrsotria fumigata	IV	A	−		34	443	3963	7.28 (<0.0001)
		B	+	120	27	496	544	
Leucophaea maderae	V	A	−		150	324	1431	2.62 (<0.0001)
		B	+	163	139	528	546	

groups of animals which, in addition, had a metathoracic femur-tibia-tarsus autotomized at the indicated times. Different autotomy times were necessary due to the varying length of the different species molting cycles. In each species, forcing animals to regenerate a limb improves the synchrony of the eventual ecdysis. This suggests that there is a relatively precise delay programmed for each type of regenerating leg. When the delay is over, all the animals are ready to molt as if they had been queued up at a gate.

Independent feedback of regenerating limbs

In addition to the added synchrony caused when one leg is autotomized if two metathoracic legs are autotomized at the same time in each animal, a substantially significant additional synchrony is achieved. This is demonstrated for two species of cockroaches in Table II. This additional synchrony due to a second autotomized limb can adequately be accounted for if it is assumed that the two feedbacks of the two regenerating limbs on the control of molting are independent of each other. In that situation, due to the laws of statistical independence, the probability of the second of two independent limbs stopping its inhibition of molting at a given time is simply the square of the probability of a single limb stopping its feedback by that time. This hypothesis is tested in an additional experiment illustrated in Figure 2. According to the independent feedback hypothesis, the cumulative molting curve for the double autotomy animals should be predicted by simply squaring the cumulative curve for single autotomy. Such a predicted curve has two properties which can be compared to the data: the mean of the distribution and its shape. The predicted line in Figure 2 visually conforms closely in both the average delay as well as the increased slope (*i.e.*, decreased variability) of the cumulative molting data for double autotomy and is statistically consistent based on the Kolmogorov-Smirnov goodness of fit test ($P > 0.10$). Whatever the signal that delays molting, it seems to be adequately described as an independent signal from each regenerating limb rather than a joint effect of the combined bulk of the regenerates.

TABLE II

Variance of molting times for double vs. *single-leg autotomized animals. The probability* (*P*) *of a chance reduction in variance due to a second autotomized limb is judged by an F-test, on pooled variances when appropriate.*

Species	Instar	Culture	Limbs Autotomized, k	Culture size, n	Variance, V_k	F = V_1/V_2 (*P*)
Blattella germanica	IV	A	1	110	77.44	1.265 (<0.005)
		B	1	110	61.78	
		C	1	110	56.10	
		D	1	110	66.16	
		A′	2	110	64.00	
		B′	2	110	48.44	
		C′	2	110	43.56	
		D′	2	110	50.69	
Byrsotria fumigata	II	A	1	157	1217	2.28 (<0.0001)
		B	2	175	533	
	III	C	1	122	1038	1.408 (<0.05)
		D	2	121	737	

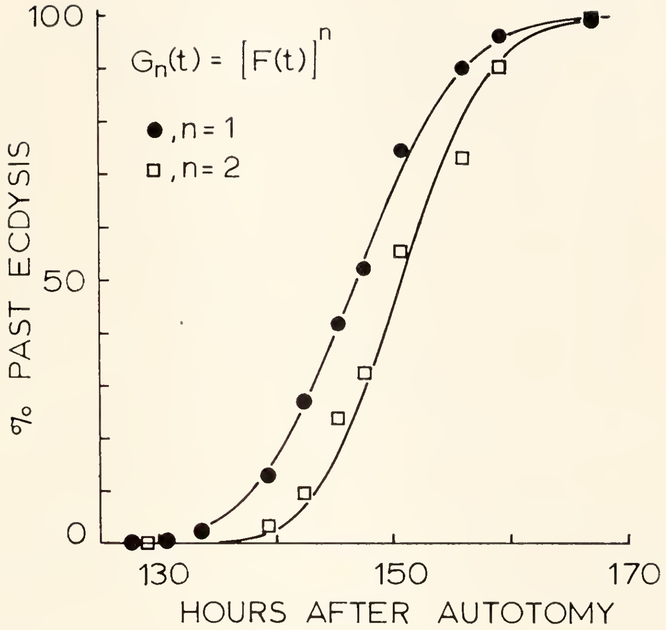

FIGURE 2. The effect of double *vs.* single autotomy on the ecdysis time in fourth instar *B. germanica.* In a culture of 104 +/or and 104 or/or larvae, the +/or larvae had 1 metathoracic femur-tibia-tarsus autotomized, while the or/or animals, had both metathoracic legs autotomized. A cumulative normal curve, G_1 (1) = F (t) was fit to the single-leg regenerate, n = 1, data and the square of the curve, G_2 (t) = $[F\ (t)]^2$, was plotted as the prediction of when the double-leg regenerates, n = 2, should undergo ecdysis.

The nature of the delay signal

The injury-metabolism model predicts that the larger the structure to be regenerated, the longer the delay. Although this correlation is true for the two leg autotomy points, confirmed by previous experiments (O'Farrell and Stock, 1953), as well as ones performed above, it is not true for nonautotomy points nor for the structures other than the walking legs of cockroaches. It has been recognized that amputation at nonautotomy points does not result in regenerates as large as does autotomy (Bulliere, 1967). The special nature of autotomy-induced delay is more clearly understood when the autotomy or amputation is performed close to the time of the critical period for regeneration (Fig. 3).

Six treatment groups served to contrast autotomy with amputation at nonautotomy points: an unamputated control group, a cercal ampuated group, tarsal and femur-tibia-tarsal autotomy groups, and tibial and coxal amputation groups. Unamputated controls showed a smooth sigmoid molting curve. Amputation of the cerci of the cockroach had no noticeable effect on the molting cycle length, and these results are not illustrated. About 15% of the tarsal and femur-tibia-tarsus autotomy groups had already passed the regeneration ciritical period at the time of autotomy and molted with the controls at the time they normally would have, without regenerating a limb; the remaining 85% of the animals of these autotomy

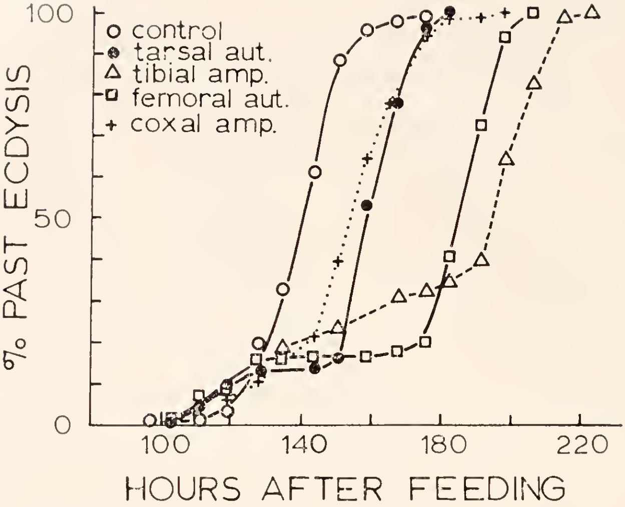

FIGURE 3. Demonstration of the stereotypy of the molting delay signal. Groups of 81 fourth instar larvae of *B. germanica* were fed for 48 hours and then an appendage was autotomized or amputated at one of the indicated levels. The pattern of ecdysis for each group is contrasted to an unautomotized control group.

groups delayed their molting cycle 25 or 55 hours, respectively, and regenerated the lost segments. Their cumulative molting curves are a composite of two sigmoid curves with a plateau in between. True amputations of limbs at non-autotomy points were performed using irridectomy scissors. Such groups of amputated animals did not conform to the previously described simple plateau and size-related delay of their cumulative molting curves. Amputation between the coxa and trochanter does not result in any regeneration at the subsequent molt. Some molting delay occurs which may be related to the substantial injury and blood loss caused by amputation at this nonautotomy point. Earlier amputation at the coxa (soon after the previous ecdysis) does result in enough time for limited regeneration from this nonautotomy point. Amputation at the femur-tibial joint also had anomalous results. According to the "injury-metabolism" model, this group should have intermediate delay between the amount for tarsal autotomy and femur-tibia-tarsal autotomy. After an initial 15% of undelayed cockroaches molted in this group, the molting curve did not plateau but continued to rise slowly until about 30% had molted; the remaining 65% delayed their molting cycles longer than the femur-tibia-tarsus regenerates. It would seem from this experiment that the severity of the injury and the amount of prospective regeneration has little to do with the amount of delay of the molting cycle. Rather it seems that the integrity of the normal route of amputation (*i.e.*, autotomy) is essential for signaling the need for an appropriate delay to allow for regeneration.

Ganglion-leg communication and molting delay

Attempts were made to demonstrate a nervous route of feedback from the autotomized limb to the brain. In these experiments the larger cockroach *P.*

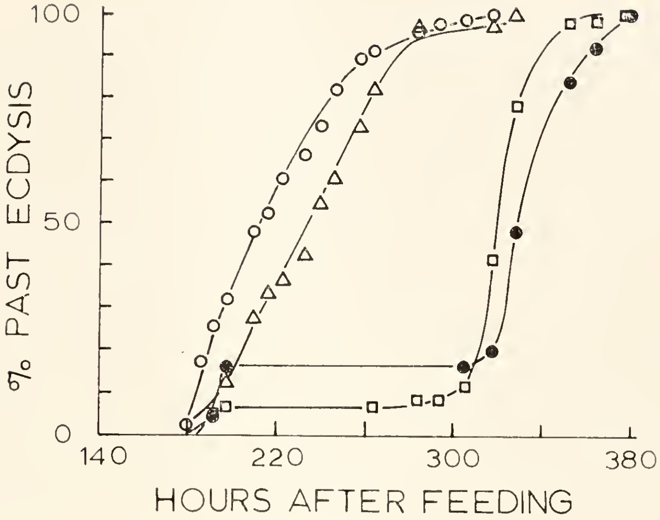

FIGURE 4. Effects of four sequentially more severe operations on cumulative molting curves of *Periplaneta americana* seventh instar larvae. The first group of 94 animals (open circle) had no leg autotomized but a sham surgical operation performed. A second group of 60 animals (square) had a limb autotomized and a sham operation also performed. The third group of 33 animals (triangle) had a limb autotomized and that leg's nerves cut. A fourth group of 25 (solid circle) had a limb autotomized, the leg nerves cut and a contralateral leg, with intact nerves, autotomized. The two plateau levels are not significantly different from one another ($P > 0.10$).

americana was used to facilitate the sectioning of nerves. Figure 4 summarizes an experiment in which information flow between the leg and its thoracic ganglion was interrupted. The four treatments were given to groups of seventh instar larvae at a time when approximately 10% of the animals had passed their critical period for regeneration.

The sham operates illustrate a normal molting cycle pattern. Autotomy resulted in a typical compound sigmoid curve with a plateau separating the undelayed nonregenerates from the delayed regenerates. Sectioning the nerves to an autotomized limb, just distal to their exit from the thoracic ganglion, eliminated the delay of molting as well as regeneration. Autotomy of an innervated contralateral leg restored the delay eliminated by nerve section. Of the 21 animals of this latter group which delayed their molting cycle to regenerate the contralateral limb, four showed partial regeneration of the nerve-sectioned leg.

The failure of nerve-sectioned autotomized limbs to delay molting and to regenerate is consistent with the nervous-feedback model. The proper sensory input from the limb stump is apparently essential to establish a delay. Since some nerve sectioned limbs did regenerate a leg, when given the proper delay time by autotomizing a contralateral limb, the ability to regenerate tissue could not in itself be sufficient to delay molting. It would seem that the connection of the stump in a formal fashion with its ganglion is essential for establishing a delay.

Ganglion-brain communication and molting delay

The ventral nerve cord between the meso- and metathoracic ganglion was chosen as a likely position to intercept information flow from a regenerating limb to the brain. Section of interganglionic connections would allow the thoracic ganglia to maintain their innervation of the legs, and therefore the centrifugal effect of nerves on regeneration would not be impaired. One complication, however, which arises in ventral nerve cord transected animals is that ecdysis cannot occur normally due to lack of coordination between the musculature anterior and posterior to the cord section. In order to bypass this difficulty, animals were assayed for regeneration by direct observation through the cuticle of the coxa (Penzlin, 1963). A culture of seventh instar *Periplaneta* nymphs was fed and the four treatments listed in Table III were administered at 84 hours after feeding, prior to the critical period for regeneration for most of the animals. Eight days after autotomy the coxae were scored for regeneration. Both mesothoracic and metathoracic autotomy groups show similar results; all animals regenerated their limb. Meso-metathoracic cord section of mesothoracic leg autotomized animals resulted in three nonregenerates even though this level of cord section should not have interfered with communication of the mesothoracic autotomy information to the brain. These animals should have delayed molting to regenerate as did the autotomy controls. The difference between the results of the cord-section controls and the pooled autotomy controls is significant by Fisher's exact test ($P = 0.02$), which suggests that cord section itself increases the propensity to delay molting and regenerate. This unexpected difference among the controls may be due to injury associated with cord section or might be related to the normal initiation of molting which may require abdominal stretch receptor information input to the brain *via* the ventral nerve cord. Isolating the brain from a ganglion innervating an autotomized limb by cutting the interganglionic connectives dramatically reduces the proportion of regenerating limbs (treatment 4 *vs.* treatment 3; Fisher exact test, $P < 0.005$), suggesting that communication *via* the ventral nerve cord between the ganglion and the brain is important for molting delay.

The relation of ecdysone pattern to regeneration

In order to test whether regeneration is actually causing a delay of brain hormone release and subsequent ecdysone secretion, it would be necessary to know the titers of these hormones in individual cockroaches during the molting cycle. Although brain hormone titers are not as yet available, the development of radioimmune assays for ecdysone have made measurements of ecdysone titers in individual animals a reality (Bollenbacher *et al.*, 1975).

In a preliminary experiment, 100 μl samples of hemolymph were obtained from groups of fourth instar larvae in synchronous cultures of *Blatta orientalis*, and ecdysone was extracted from these samples. Both 5 and 50 μl equivalents of the hemolymph extracts were assayed for ecdysone content. Ecdysone was reliably measured at each of the four chosen times during the cockroach molting cycle, using either the 5 or 50 μl aliquots. This confirmed the adequacy of our assay technique and suggested that 5 μl samples of hemolymph from individual animals could be used to estimate ecdysone titers in animals from synchronized cultures.

In the next experiment a large group of sixth instar *Blatta orientalis* nymphs were synchronized by feeding. Every 24 hours a 5 μl sample of blood was taken from each of five randomly selected animals. At 98 hours half of the remaining animals had a metathoracic femur-tibia-tarsus autotomized, and samples were subsequently taken from both unautotomized controls and autotomized animals. Figure 5 demonstrates that, in the control nonregenerating animals, a peak of ecdysone occurs in the later third of the molting cycle with the titer of ecdysone falling to low levels by the time of ecdysis. This is similar to earlier observations using the *Calliphora* bioassay for ecdysone in extracts of pooled whole *B. germanica* (Masner, Hangartner, and Suchy, 1975). The regenerating group shows a substantial delay in the peak of ecdysone parallel to the delay in molting. The five individual titers comprising each mean during the peak days of ecdysone titer in both groups show a substantial ecdysone concentration, suggesting that a broad peak of ecdysone titer occurs rather than transitory increases in a few individuals.

Ecdysone titer and the regeneration critical period

In order to clarify the relationship of the regeneration critical period to the increasing titer of ecdysone during the molting cycle, an experiment was designed in which the two phenomena were measured simultaneously in the same animals. Care was taken to estimate the limits of sensitivity of the radioimmune assay in order to establish whether any increase in ecdysone titer precedes the major rapid increase illustrated in the previous experiment. Males and females were analyzed separately, since they are known to differ in molting cycle length at this instar.

The data on regeneration critical period and individual ecdysone titer illustrated in Figure 6 can be read and interpreted in various ways. It should be noted that the midpoint of the males distribution of regeneration critical periods occurs about one day later than the females.

An increase in ecdysone titer seems to occur associated with passing the regeneration critical period. This is suggested by the fact that the proportion of animals with ecdysone titer greater than 0.25 ng per microliter never exceeds the proportion of animals past the regeneration critical period. Also, the major peak of ecdysone titer in the females is just starting on day ten in this experiment, while already on days seven and eight there are five of twenty animals with titers exceeding 0.6 ng per microliter. These five animals are not likely to be outliers of the major ecdysone peak but more likely represent animals exhibiting a transitory increased ecdysone titer associated with passage of the regeneration critical period. Of course, the precise pattern of titer changes will remain moot until it is possible to follow an individual animal through the entire period of ecdysone titer change.

Circumstantial evidence for levels of ecdysone action

Ecdysone inhibits regeneration and molting delay when injected into autotomized cockroaches (Bulliere, 1972; Kunkel, 1975b). The timing of ecdysis due to exogenous ecdysone can vary considerably depending upon the dose. Fifth instar *Blatta orientalis* larvae had a metathoracic femur-tibia-tarsus autotomized 95 hours after feeding. The autotomized animals were randomized into six groups of 25, and doses of ecdysone were administered. The response to ecdysone for

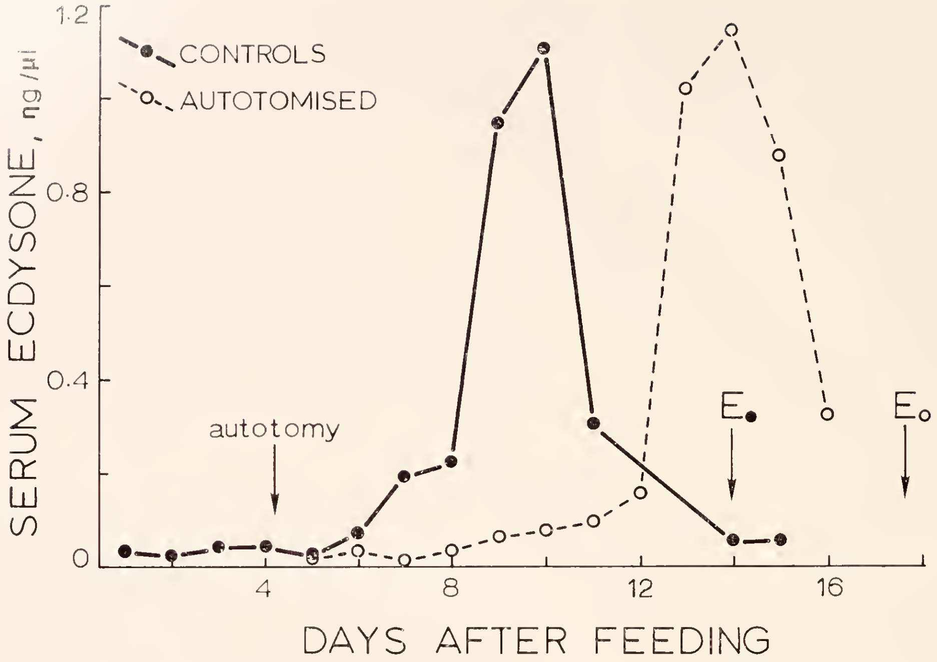

FIGURE 5. Serum ecdysone levels in normal and autotomized sixth instar larvae of *Blatta orientalis*. Autotomy of a metathoracic femur-tibia-tarsus was performed at the indicated time. A five microliter sample of hemolymph was taken from each of five animals randomly selected from mass synchronized cultures. Ecdysone was measured on individual blood samples by radiommune assay. The average ecdysone titer for each day is plotted for each group. The average times of ecdysis, E, of the donor cultures are indicated by arrows.

each animal has an all or none component; that is, either an animal delays its molting cycle and regenerates a limb or it neither delays molting nor regenerates (Kunkel, 1975b). In addition, the animals which are prevented by the exogenous ecdysone from delaying their molting cycle to regenerate exhibit graded dose dependent responses to the injected ecdysone (Fig. 7). Below 1 µg/animal, no animals showed any response to ecdysone. At the lower doses of 1 and 2 µg/animal, the larvae which did not delay their molting cycles underwent ecdysis beginning at the same time as the unautotomized controls. At the higher doses, 4 and 8 µg/animal, the larvae undergo ecdysis at successively earlier times, such that at the 8 µg/animal dose the median time of ecdysis is 1.6 days earlier than the unautotomized controls. This ability to accelerate the timing of ecdysis to different degrees is circumstantial evidence that the action of ecdysone is spread over a substantial time period in the individual cockroach. The low doses of ecdysone may have an effect comparable to the suggested early peak or rise of ecdysone which follows the brain critical period. The higher doses of ecdysone may be having an effect comparable to the major peak of endogenous ecdysone, bypassing or compressing the earlier events of the molting cycle, as has been described in the giant silk moth (Williams, 1968).

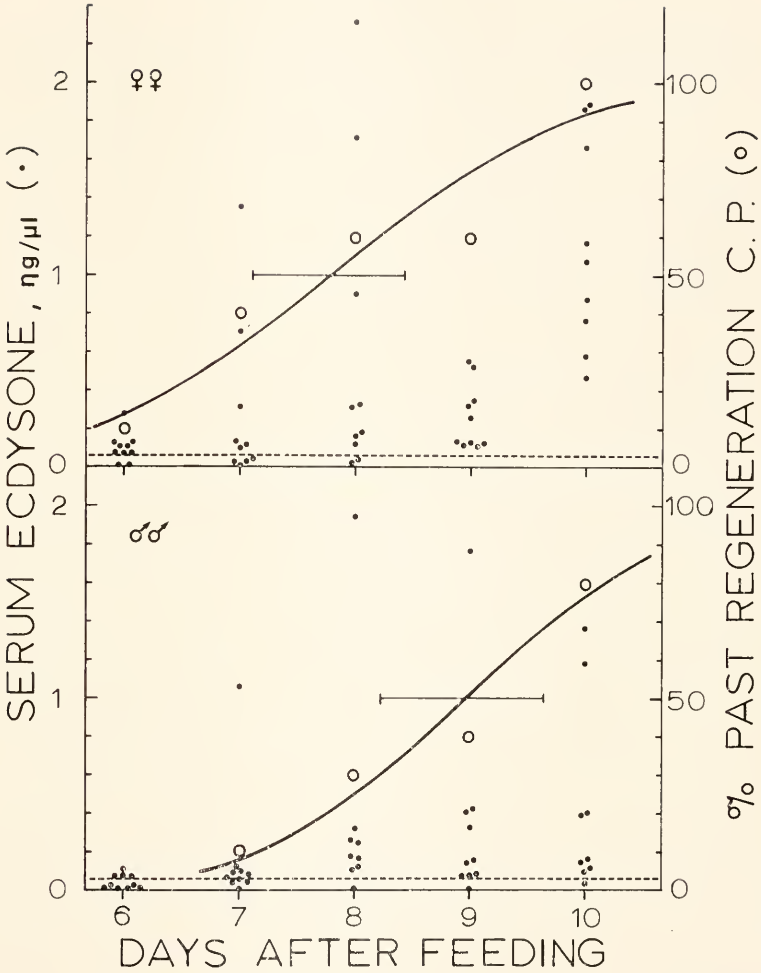

FIGURE 6. Simultaneous ecdysone titer and regeneration critical period determination for sixth instar larvae of *Blatta orientalis*. Twenty larvae, ten of each sex, were randomly selected from a large synchronized culture on days 6 through 10 after feeding. Each animal had a metathoracic femur-tibia-tarsus autotomized and a five microliter sample of hemolymph taken for ecdysone assay. Individual ecdysone titers (solid circles) and the proportion of animals which did not regenerate (open circles), are plotted against the time of autotomy and blood sampling. A cumulative normal curve was fitted to the proportions and used to calculate a 95 per cent confidence interval for the mean time of the regeneration critical period (horizontal bar). A 95 percent one sided confidence limit of the sensitivity of the ecdysone assay is indicated by the dashed line in each graph.

DISCUSSION

In the present study an attempt is made to clarify how limb regeneration in cockroaches causes a delay of the molting process. Evidence is presented which

Table III

Role of the ventral cord route between ganglion and brain in the autotomy-induced molting cycle delay and leg regeneration.

Treatment	Number of nonregenerates	Number of regenerates	Number dying
1. Mesothoracic autotomy control	0	15	0
2. Metathoracic autotomy control	0	15	0
3. Mesothoracic autotomy + meso-meta cord section control	3	9	3
4. Metathoracic autotomy + meso-meta cord section	11	2	2

contradicts a previous "injury-metabolism model" and additional evidence is presented which supports a "nervous-feedback model" of molting delay.

The "injury-metabolism" model of molting delay was based on the results of a number of workers who observed correlations between the length of delay and the size of a regenerate (O'Farrell, Stock, Rae and Morgen, 1960; Pohley, 1965). Testing this model depended on deciding a direction of causality which underlies the observed correlation of size with delay-length. The experiments carried out above demonstrated that the amount of regenerating tissue could not be causing the length of the delay. Most likely there is an average delay time programmed for each type of autotomy in the hemi-ganglion serving a leg. This programmed delay was shown above not to depend on the size of the autotomized structure nor on the size of the resulting regenerate. That delay length is independent of the mass of the regenerating tissue is the major reason for rejecting the injury-metabolism model of molting delay. In lieu of the injury-metabolism model of delay and since the brain critical period had been mapped close to the time of the regeneration critical period (Kunkel, 1975a), it was hypothesized that the regenerate was in some way preventing brain hormone release. Consequently, routes of feedback to the brain were investigated. Despite the fact that innervation is not essential for regeneration to proceed if autotomy occurs early in a molting cycle (Penzlin, 1964), it was demonstrated above that the ganglion of the autotomized limb must be connected to the limb for proper delay of molting to occur, particularly when autotomy takes place when the regeneration critical period is imminent. Moreover, the ganglion of the autotomized limb must maintain its connection with the brain in order to establish the delay of molting. It is strongly suggested that the regenerating limb is delaying molting by preventing brain hormone secretion.

The nature of the source of the delay signal is subject to speculation. It may be that the sensory input from the limb is the source of information about the integrity of the leg. The pattern of leg movements in cockroaches is immediately responsive to autotomy of a limb (Delcomyn, 1971). Interestingly, like molting delay, walking pattern does not change when a leg is amputated at the femoral-tibial joint, a nonautotomy point. If the information on integrity of the locomotory system were able to be monitored by the brain, then a nervous route of control of brain hormone release would be a reasonable model for molting delay. If the limb were autotomized after the brain had released its ecdysiotropin, the animal

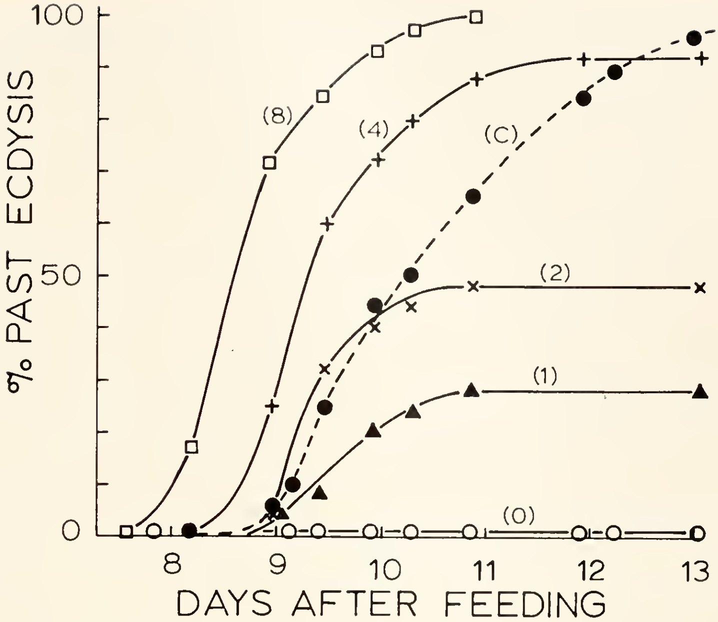

FIGURE 7. A graded response of cockroaches to ecdysone. Graded doses of ecdysone (1, 2, 4, and 8 μg/animal; triangle, X, cross, square, respectively) were injected into groups of 25 limb autotomized test animals. The cumulative percentage of each group which molted by a given time is plotted. In a control autotomized group injected with saline (open circle) 76 out of 77 had not molted by day 13. An unautotomized control group (solid circle) of 100 animals is included to show the normal time of molting.

would already be committed to molt and the resulting rising titer of ecdysone would shut off any regeneration which started. Thus, the all or none nature of regeneration in cockroaches is explained.

The length of the delay of molting was shown previously, as well as in the present study, to be specific for each type of regenerate and thoracic segment. The simplest explanation of the control of the length of delay is that it is determined by the hemiganglion serving each leg. It was shown above that the length of the delay associated with each regenerating limb is independent of each other regenerating limb. This suggests that there is an independent delay-determining process in the hemiganglion serving each leg. This might involve the neurons whose synthetic activities have been shown to be activated during regeneration (Cohen and Jacklet, 1965), or those which play an active part in the coxal muscle regression during limb regeneration (Shapiro, 1976). Differences in the neurons affected by tarsal and femur-tibia-tarsal autotomy could control the observed difference in delay due to level of autotomy.

The end of the delay associated with each leg and level of autotomy independently releases the brain from inhibition and when all autotomized limbs have stopped their delay signals, the brain promptly initiates a molting cycle. This ordered release from molting inhibition results in considerable increases in synchrony of molting cycles in the diverse selection of cockroach species examined. Although the additional increase in synchrony with multiple autotomy is in this instance a fortuitous phenomenon having little or no obvious functional value to the cockroach, the concept of poorly-timed phenomena combining in an ordered way to produce a more precisely-timed phenomenon is a valuable concept that could have applications to many areas. It is of practical use in working with cockroaches, because it can produce a culture of developing animals with substantially increased synchrony.

The end of the brain's delay in initiating molting must involve the final secretory release of ecdysiotropin, which has been demonstrated to stimulate the prothoracic glands to produce ecdysone (Gersh and Sturzebecker, 1970). However, the pattern of ecdysone secretion following the brain critical period is not simple. The major peak of ecdysone in cockroaches occurs substantially after the regeneration critical period in the last third of the molting cycle. There is, however, evidence reported above that there is a low level or possibly a transitory high ecdysone titer which occurs about the time of the regeneration critical period. The timing of the major peak of ecdysone would place it approximately at the time of apolysis. The period of epidermal proliferation follows the brain critical period but precedes the major peak of ecdysone (Kunkel, 1965). Since the molting cycle becomes independent of the brain after the brain critical period, it would seem that the brain hormone stimulates the prothoracic glands to produce the early rise of ecdysone, and once this has occurred the rest of the events of the cycle occur independently of the brain. Part of the molting schedule, including epidermal proliferation, must be stimulated by low titers or early transitory increases of ecdysone and the rest of the molting schedule from apolysis on is controlled by the major ecdysone peak. A sequential action of ecdysone in controlling the molting cycle may be responsible for the observed differences in timing of molting seen when different doses of ecdysone were used to overcome the regeneration-induced delay of molting. This pattern of ecdysone titer in cockroaches is reminiscent of the two peaks of ecdysone seen during pupal development in the tobacco hornworm (Truman and Riddiford, 1974), but the pattern in cockroaches apparently does not require continuous modulation by the brain.

I am grateful to Professor Michael Locke for providing the stimulating atmosphere in which this research was initiated. The work was supported by grants from the National Institutes of Health, GM-09960 to M. Locke and GM-33259 and AI-11269 to J.G.K.

SUMMARY

1. The amount of regenerating limb tissue does not control the length of molting delay in cockroaches; rather, a programmed delay associated with each autotomy level and segment allows an appropriate delay for accomplishing the necessary regeneration.

2. Delay of molting is accomplished by inhibiting ecdysiotropin release.

3. Each regenerating limb produces a delay independent of other regenerating limbs.

4. Single and simultaneous double autotomies can be used to obtain substantially increased synchrony of the molting cycle of a wide variety of cockroach species.

5. The signal to delay molting is transmitted to the brain along the ventral nerve cord and requires the stereotyped sensory input associated with autotomy to initiate it.

6. There are two phases of ecdysone titer increase during the molting cycle of cockroaches. An early transitory or gradual increase is associated with the regeneration critical period, while a later major peak is associated with apolysis. The major ecdysone peak was shown to be delayed during leg regeneration.

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THE EFFECT OF SALINITY UPON PHOTOTAXIS AND GEOTAXIS IN A LARVAL CRUSTACEAN

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Vertical movements by pelagic larvae of marine benthic invertebrates are directed by light and gravity. The spatial orientations of these responses are known to be affected by light intensity, temperature, hydrostatic pressure, feeding, and salinity (reviewed by Forward, 1976a). This last parameter, which is highly variable in coastal plain estuaries, can alter both phototactic and geotactic behavior.

A negative phototaxis can be induced in normally photopositive larvae of various estuarine organisms by a sudden exposure to low salinities. The salinity change necessary for such a phototactic reversal may range from 4.5% to 66% dilution of seawater (Edmondson and Ingram, 1939; Lynch, 1949; Lyon, 1906; Ranade, 1957). Furthermore, Edmondson and Ingram (1939) found that barnacle nauplii regained positive phototaxis in ten minutes after a salinity decrease. Evidence that an increase in salinity alters the sign of phototaxis is unreported.

A negative geotaxis enables larvae to remain up in the water column in the absence of light by directed swimming which compensates for a tendency to sink (Sulkin, 1973). The depth in the water column at which larvae are found, however, is related to developmental stage. Generally, later larval stages remain lower than earlier stages (*e.g.,* Bousfield, 1955; Carriker, 1951; Lynch, 1947; Sandifer, 1975; Sulkin, 1973).

Work with salinity discontinuities in nature (Grindley, 1964) and in the laboratory (Harder, 1968; Lance, 1962; Lyster, 1965; Roberts, 1971; Scarratt and Raine, 1967), as well as laboratory experiments with fluctuating salinities (Haskin, 1964; Hughes, 1969; Hughes and Richard, 1973), have shown that a downward movement is the common response to a salinity decrease. Likewise, an increase in salinity will induce an upward movement (Haskin, 1964; Hughes and Richard, 1973). Little attempt, however, has been made to determine whether such movements result from a response to light and/or to gravity.

The present study examines the effect of sudden salinity changes upon phototaxis and geotaxis in larvae of the brachyuran crab *Rhithropanopeus harrisii* (Gould). This species was chosen for study because its larvae occur in coastal plain estuaries, where they are subjected to natural salinity variations. In addition, much is known about the larvae: the effects of salinity on larval development (Costlow, Bookhout, and Monroe, 1966), osmoregulatory ability (Kalber and Costlow, 1966), the ontogeny of phototaxis (Forward, 1974; Forward and Costlow, 1974), the shadow response (Forward, 1976b), the effect of temperature on phototaxis and geotaxis (Ott and Forward, 1976), polarotaxis (Via and Forward,

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1975) and occurrence of the larvae in estuaries (Bousfield, 1955; Chamberlain, 1962; Pinschmidt, 1963; Sandifer, 1975).

The results from the study indicate that sudden salinity changes alter phototaxis and geotaxis by Stage I and IV zoeae, and it is suggested that these behaviors contribute to vertical movements within a stratified estuary.

MATERIALS AND METHODS

Ovigerous female specimens of *Rhithropanopus harrisii* (Gould) which were collected from the Neuse River in eastern North Carolina, from May until August, were maintained in either 10 or 20‰ filtered sea water. Upon hatching, larvae were cultured using techniques similar to that of Costlow *et al.* (1966) in 10 or 20‰ filtered sea water (acclimation salinities) and maintained at 25° C on a 12L:12D cycle in a Sherer Controlled Environment Chamber (Model CEL-44). Larvae were transferred daily to clean finger bowls containing fresh sea water and were fed newly hatched *Artemia salina* nauplii.

The light source for the phototaxis experiments was a La Belle slide projector fitted with a 300 W tungsten bulb. Light was filtered by two hot mirrors (Baird Atomic) and a Corning No. 1–75 filter in order to reduce heat, and was then filtered to 500 nm with a thin film absorption filter (half band pass 7.4 nm; Ditric Optics, Inc.). This wavelength was chosen for experimentation since a previous study indicated that this species shows maximal sensitivity at this spectral region (Forward and Costlow, 1974). There is no evidence that larvae of *R. harrisii* exhibit color dances or other changes in swimming orientation with wavelength, as has been reported in some crustaceans (*e.g.*, Dingle, 1962). Light intensity was regulated by neutral density filters (Ditric Optics, Inc.) and was measured by a radiometer (YSI model 65).

Phototactic behavior of larvae of *R. harrisii* was monitored in both a horizontal and a vertical plane upon sudden exposure to a range of different salinities. Experimentation using a horizontally-directed light source was performed with a 15 × 3 × 3 cm leucite cuvette, which was divided into five equal sections along the longitudinal axis. The sections were separated by thin slides constructed so that all could be moved vertically in unison.

The test chamber with the vertically directed light source was a 45 × 8 × 7.5 cm upright rectangular leucite cuvette. Light entered the chamber from either above or below by means of an appropriately positioned mirror. The cuvette was marked into ten vertical sections, each 3.8 cm in height. A 20 W daylight fluorescent light was mounted vertically behind the chamber to aid in viewing larval positions at the termination of stimulation.

During larval development *R. harrisii* has four zoeal stages which are free-swimming in the plankton and are phototactic. The subsequent megalopa stage probably settles out of the plankton and is unresponsive to light (Forward, 1974; Forward and Costlow, 1974). Thus only Stage I and IV zoeae were tested, since they are responsive to light and any ontogeny in responses should be apparent. For all experiments larvae were light-adapted under a 60 W incandescent bulb in addition to fluorescent room lights, for at least one-half hour prior to testing. Experimentation was performed within a six-hour period which began three hours after the onset of the light phase in order to avoid any complications due to a biological rhythm in either phototaxis or salinity responses. Testing was performed at room temperature (22–25° C). Even though this temperature range does deviate from that in the culture cabinet, the change is insufficient to alter either phototaxis or geotaxis (Ott and Forward, 1976). For each phototactic determination with the horizontal and vertical light source approximately 60 larvae from at least three separate females were used. Salinities for most experiments were measured with an American Optics refractometer (accuracy 1.0‰). However, the salinities of the solutions used for determining the salinity threshold for a negative phototaxis were measured with an osmometer (Model 65–31, Advanced Instruments, Inc.; accuracy 0.2‰), the calibration for which was established with an induction salinometer (Hytech Model 6220).

The general procedure for horizontal stimulation consisted of pipetting ten larvae into the center section of the cuvette filled with sea water of the experimental salinity. After 50 seconds in total darkness, the slides separating the sections were raised, and 10 seconds later the light stimulus was applied for a duration of 30 seconds, after which time the slides were lowered. This stimulus time was chosen because preliminary testing indicated that it was sufficient for initiating a clear response, but was insufficient to induce an apparent total (100%) response. The distribution of larvae among the five sections was then recorded. Thus, larvae were stimulated with light one minute after exposure to sea water of the experimental salinity, and the light stimulus was terminated 1.5 minutes after exposure to the sea water. Controls of random swimming in darkness were also run following a similar procedure, except that larvae remained in darkness for the entire trial. For determining phototactic responses, only larvae located in the section nearest the light source were considered to be displaying positive phototaxis, while only larvae in the furthest section were said to show a negative response. Levels of responsiveness under different conditions were statistically compared by determining a Z-statistic for testing the difference between two proportions (Walpole, 1974) and significant differences tested at the 0.05 level.

Initially, the phototactic responses of larvae acclimated to 20‰ sea water and stimulated in a horizontal plane were measured for stimulus intensities of 1.93 to 1.93×10^{-5} $\mathrm{Wm^{-2}}$, as well as a control in darkness. The experimental salinities were 5, 20, and 40‰. To establish the smallest decrease in the salinity from the acclimation salinity that would evoke a reversal in the sign of phototaxis from positive to negative, larvae were acclimated to 10 or 20‰ sea water. They were then exposed to salinity decreases of 0.8, 1.1, 1.3, 1.5, 1.7, 2.0‰, and levels of positive and negative phototaxis were observed upon stimulation at 0.19 $\mathrm{Wm^{-2}}$, an intensity which at the acclimation salinity provokes a positive response.

Further experiments measured the time for a positive phototaxis to be reestablished. Larvae were exposed to salinity decreases equal to or slightly greater than threshold values for inducing a reversal in phototactic sign. For comparison, Stage I zoeae (acclimation salinity 20‰) were subjected to 5‰ and a similar time measured. The testing procedure was similar to that previously delineated, in that the light stimulus ($I = 0.19$ $\mathrm{Wm^{-2}}$) commenced one minute after exposure to the salinity change. Then, with the light remaining on, the distribution of larvae in the test cuvette was noted 30 seconds later and thereafter at one minute intervals until a 45–55% response level of positive phototaxis was established. This level

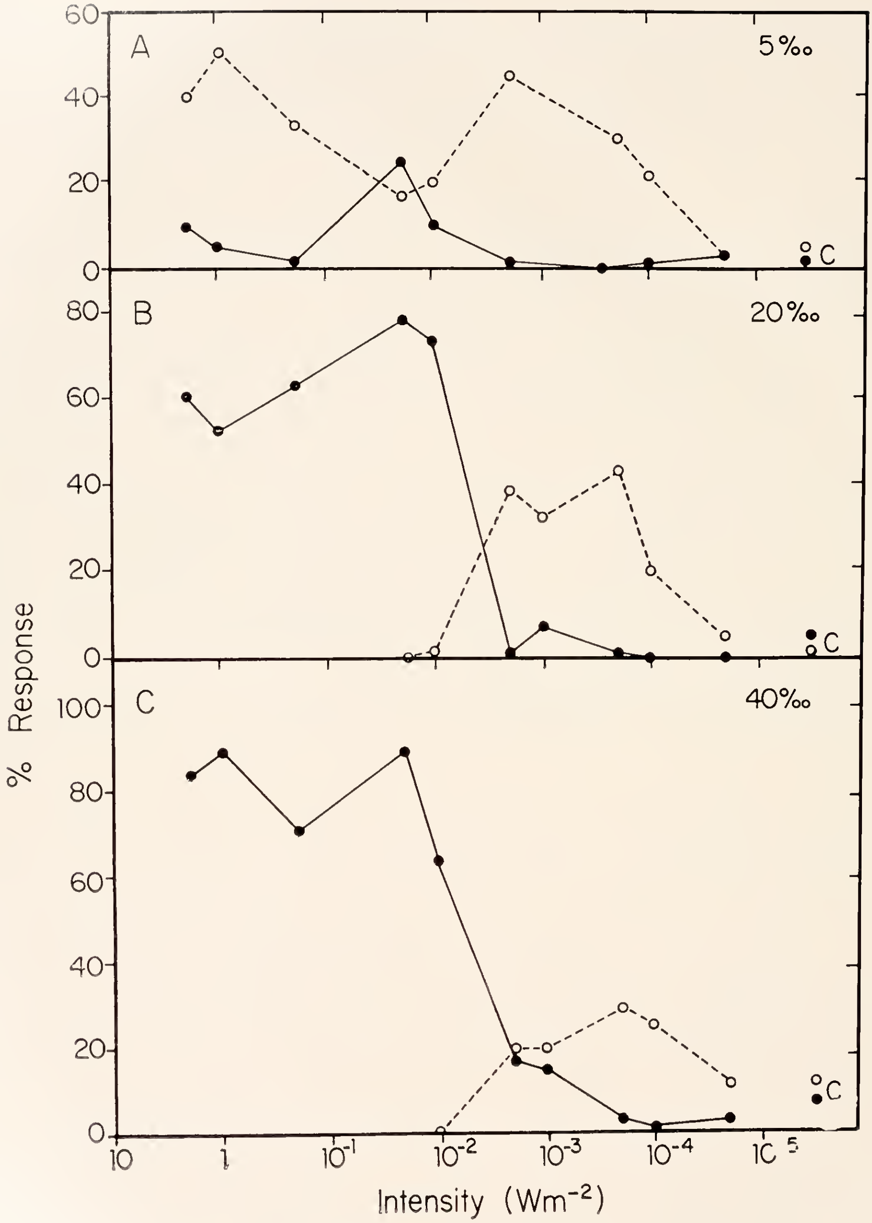

FIGURE 1. Stage I: per cent response (ordinate) of positive (solid dot-solid line) and negative (open dot-dashed line) phototaxis to various stimulation intensities of 500-nm light (abscissa) upon sudden exposure to salinities of 5‰ (A), 20‰ (B), and 40‰ (C) for larvae acclimated to 20‰. Random swimming in the positive (solid circle) and negative (open circle) direction was determined with no stimuli present. Sample size for each point was 60 individuals. In 5‰ sea water (A), the level of positive phototaxis at all light intensities above 10^{-3} Wm^{-2} is significantly less than that in 20‰. The level of negative phototaxis present at low light

of responsiveness was chosen to indicate a total return of responsiveness, since it is a level similar to that shown by the larvae before they experience the salinity change. The amount of time needed to reach this "total" recovery and a level of responsiveness that was one half this value (50% recovery) were determined.

Experiments done in the vertical plane were designed to test the effect of sudden salinity changes upon vertical distributions. Larvae were tested in darkness and upon vertical stimulation with a light intensity of 1.5×10^{-1} Wm^{-2} as measured at the surface of the column nearest to the stimulus source (*i.e.*, bottom of column with light from below, top of chamber with light from above). This intensity induces a positive phototaxis at acclimation salinities when the larvae are stimulated horizontally. Larvae were acclimated to 20‰ sea water, and a range of seven salinities were tested: 5, 10, 15, 20, 25, 30, and 40‰.

For testing, larvae were pipetted 10 cm down into the cuvette filled with the experimental salinity sea water. They were then either subjected to the light stimulus or allowed to remain in darkness for two minutes, after which time the fluorescent light mounted behind the chamber was turned on, and the distribution of larvae recorded. Preliminary studies indicated that this stimulus duration gave larvae more than sufficient time to reach equilibrium positions. Distributions in light and darkness at either the top or bottom section of the column at each salinity were compared statistically by means of a 2 × 2 contingency test in which larvae were assigned to either of two groups; top or bottom and all others. Differences were tested at the 0.05 level. Negative geotaxis was considered vertical movement up in the water column in darkness, while positive geotaxis was the opposite.

The passive sinking rates of larvae were measured in 5, 10, 15, 20, and 40‰ at room temperature. Larvae were anesthetized by placing a few drops of propylene phenoxytol in a well slide containing 10 to 15 larvae in water at the acclimation salinity (for methods see Forward, 1976a). Larvae were gently placed in the upright cuvette filled with the experimental salinity sea water and allowed to sink at least 7.6 cm (two sections) in order to reach terminal velocity. The time it took larvae to traverse a subsequent 3.8 cm was measured by a stopwatch. Sinking rates were measured for at least 20 separate animals under each salinity condition. In addition, descent rates and swimming behavior of unanesthetized larvae (20‰ acclimation salinity) were examined in 5, 10 and 15‰ salinity within the vertical column with light entering from above. A procedure similar to that for anesthetized larvae was followed, and the light stimulus intensity was 0.15 Wm^{-2}. Using the Student's *t*-test, mean rates for normal and anesthetized larvae at each salinity were tested for significant differences at the 0.05 level.

RESULTS

Horizontal testing

The pattern of phototaxis by Stage I and IV zoeae of *Rhithropanopeus harrisii* is altered upon exposure to low salinity sea water. Stage I zoeae sub-

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intensities (less than 10^{-2} Wm^{-2}) in 40‰ sea water is significantly greater than control levels of random swimming but is significantly less than similar responses in 20‰. At light intensities above 10^{-3} Wm^{-2}, the level of positive phototaxis in 40‰ is significantly greater than that in 20‰ except at 0.2 Wm^{-2}, where the responses are not significantly different.

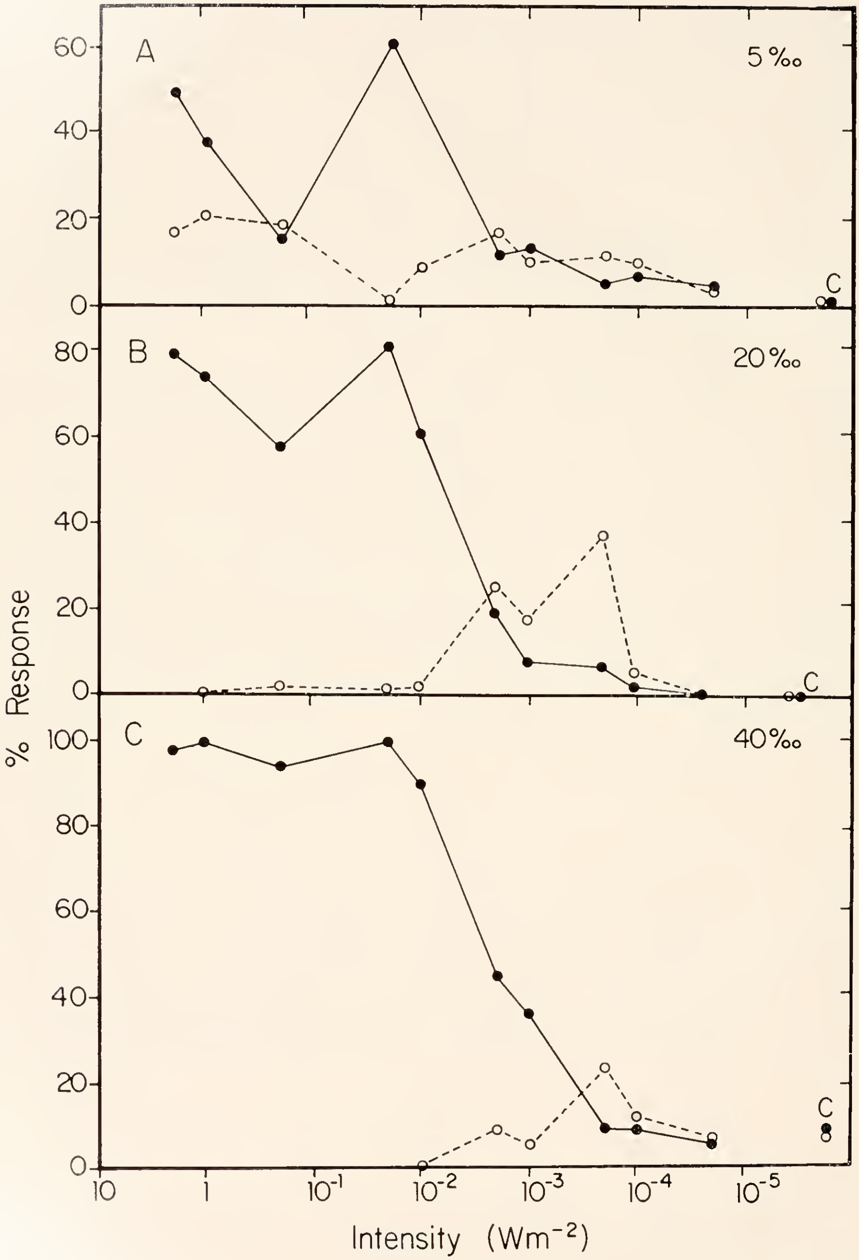

FIGURE 2. Stage IV: per cent response of positive and negative phototaxis to various intensities of 500-nm light upon sudden exposure to salinities of 5‰ (A), 20‰ (B), and 40‰ (C) for larvae acclimated to 20‰. The figure is plotted as described for Figure 1. Sample size for each point was 60. At the intensity (1.9×10^{-4} Wm^{-2}) that induces the greatest

jected to 5‰ (Fig. 1A) display a pronounced negative phototaxis at light intensities above 10^{-3} Wm^{-2}, while the normal positive response, which is prominent at the acclimation salinity of 20‰ (Fig. 1B), is suppressed. Such a dramatic reversal in phototaxis is not observed upon exposure to 40‰ sea water (Fig. 1C), where the response pattern is similar to that at 20‰. The level of positive phototaxis to intensities above 10^{-2} Wm^{-2}, however, is generally greater for larvae in 40‰.

Stage IV zoeae, subjected to 5‰ (Fig. 2A), also show greater negative phototaxis at high light intensities (above 10^{-2} Wm^{-2}) than those observed at 20‰ (Fig. 2B). In addition, while levels of positive phototaxis are not as great as those at 20‰, a positive response is still present at intensities above 10^{-2} Wm^{-2}. Like Stage I, the pattern of photoresponses of Stage IV zoeae exposed to 40‰ is similar to that seen at the acclimation salinity (Fig. 2C). The negative phototaxis at low light intensities, however, is suppressed, and the level of positive phototaxis at higher intensities is greater.

Since lowering the salinity generally reverses the sign of phototaxis from positive to negative at stimulus intensities above 10^{-2} Wm^{-2}, further experiments established the amount of salinity decrease from the acclimation salinity necessary for this change. The threshold value for inducing a reversal in phototactic sign is considered to be that salinity which produces a negative response level significantly greater than the control level in the acclimation salinity. When larvae are acclimated to 20‰ salinity, the threshold values are: Stage I zoeae 1.1‰, and Stage IV zoeae 2.0‰ (Fig. 3A, B). Upon acclimation to 10‰ the threshold value is 1.3‰ for both Stages I and IV zoeae (Fig. 3C, D). Since these values are similar, the threshold is apparently independent of the acclimation salinities and larval age.

The length of time for recovery of a positive phototaxis upon lowering the salinity is also independent of the acclimation salinity and developmental stage, as well as the magnitude of the salinity decrease (Table I). A total recovery occurs in approximately 5.5 minutes under all conditions, with a 50% recovery apparent in 1.5–3.2 minutes. Clearly the suppression of a positive phototaxis is a short-term phenomenon.

Throughout the experiments with horizontal stimulation, qualitative observations were made of larval behavior. While elimination of a vertical component in responses had been sought, apparently this could not be totally avoided. Larvae exposed to 5‰ were consistently found on the bottom of the cuvette, while larvae in 40‰ sea water swam freely. To quantify these observations and investigate the interaction of phototactic and geotactic components of a response to salinity stimuli, tests were performed in a vertical column.

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negative response in 20‰ (B), the response level in 5‰ (A) is significantly less. At higher intensities (above 10^{-2} Wm^{-2}), the level of negative phototaxis is significantly greater than that in 20‰. Levels of positive phototaxis at intensities above 10^{-2} Wm^{-2} are significantly greater than the dark control, yet significantly less than those in 20‰. In 40‰ (C) sea water, the only intensity at which a level of negative phototaxis is significantly greater than the control is 1.9×10^{-4} Wm^{-2}, but the level at this intensity does not differ significantly from that in 20‰. At intensities of 10^{-3} Wm^{-2} and greater, the level of positive phototaxis in 40‰ is significantly greater than that in 20‰.

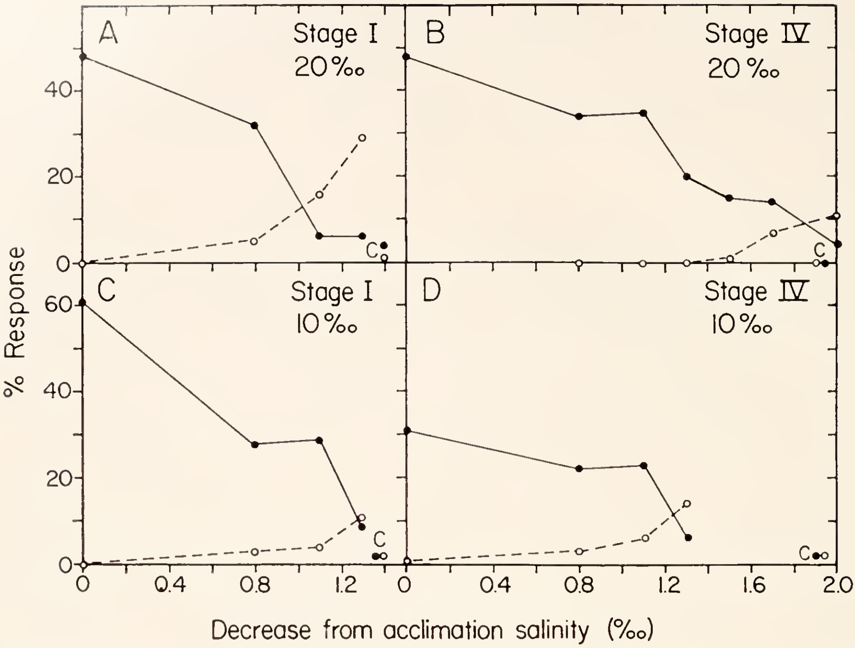

FIGURE 3. The minimum salinity decrease necessary for reversing the sign of phototaxis. Per cent response (ordinate) of positive (solid dot-solid line) and negative (open dot-dashed line) phototaxis to a stimulus of 0.19 Wm^{-2} 500-nm light upon various salinity decreases from the acclimation salinity (abscissa) by Stage I (A) and IV (B) zoeae acclimated to 20‰ and Stage I (C) and IV (D) zoeae acclimated to 10‰. Random swimming in the positive (solid circles) and negative (open circles) directions was determined with no stimulus present. The average sample size for each point was: (A) = 77; (B) = 65; (C) = 95; and (D) = 80.

Vertical testing

Experiments with light entering the column from above resembled the downward-directional aspect of natural light conditions and examined the effect of salinity changes on vertical distributions under this irradiation regime. Those with light entering the column vertically from below had two objectives. The first was to further clarify the role of a negative phototaxis in establishing vertical distributions upon sudden exposure to salinities below the acclimation salinity. The second was to determine whether the positive phototaxis or negative geotaxis is the dominant behavioral response during the ascent observed upon exposure to salinities above the acclimation salinity.

The vertical distribution of larvae is age-dependent. In acclimation salinity sea water, Stage I zoeae (Fig. 4) are dispersed throughout the vertical column, although slightly more are present in the lower half (light from above 67%, darkness 68%; no significant difference). Stage IV zoeae, however, are positioned lower in the column (Fig. 5). In darkness, essentially all larvae (97%) were found in the lower half of the column, while 85% of those irradiated from above were so distributed.

Table I

Times to recover positive phototaxis (total recovery) and responsiveness at one-half this value (50% recovery) to 500-nm light of intensity 0.19 Wm^{-2} by Stage I and IV zoeae acclimated to 10 and 20‰ sea water and exposed to lower salinities (Salinity). Times were measured from the initial exposure to the salinity decrease, and response (%) indicates the level of positive phototaxis at the time recorded. Average n was 76.

	Total Recovery			50% Recovery	
	Salinity (‰)	Response (%)	Time (min)	Response (%)	Time (min)
20‰ acclimation					
Stage I	18.7	53	5.5	27	2.9
	5.0	53	5.0	27	3.2
Stage IV	17.5	54	5.5	27	1.5
10‰ acclimation					
Stage I	8.7	45	5.5	23	2.0
Stage IV	8.7	47	5.5	24	2.7

Experiments indicate that the vertical distribution of larvae is altered by salinity. For Stage I zoeae (Fig. 4), when the distributions in the top and bottom sections of the column are compared between the two conditions of darkness and irradiation from above, there is no statistical difference. For each test at salinities less than the acclimation salinity, Stage I zoeae are found lower in the column as compared to distributions in 20‰. This is most clearly observed at the lowest test salinity (5‰), in which 93% of the larvae in light and 92% in darkness are located at the bottom of the column (Fig. 4). Exposure to salinities above the acclimation salinity causes an upward movement. At 40‰, all larvae are positioned within the upper third of the column with approximately 97% in light and 100% in dark found in the uppermost 3 cm.

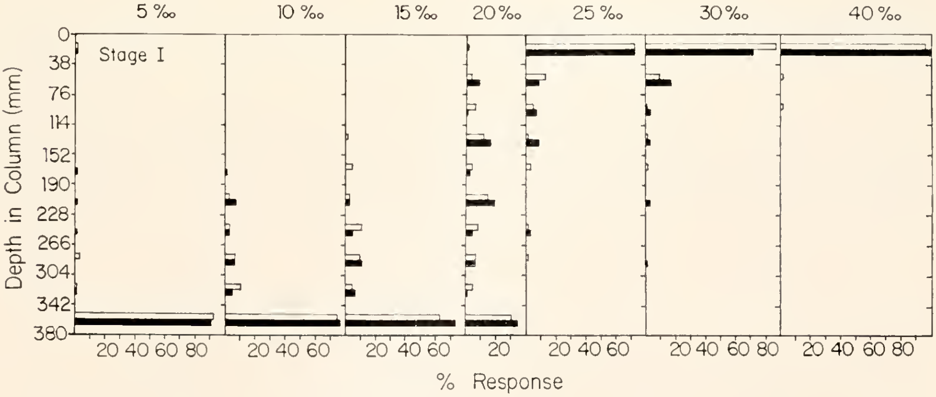

Figure 4. Stage I: percentage distribution of larvae (abscissa) according to depth in column (ordinate) upon stimulation by 0.15 Wm^{-2} 500-nm light direction from above (open bar) or in total darkness (solid bar) when acclimated to 20‰ and exposed to salinities between 5 and 40‰. Sample size for each condition was 60 individuals.

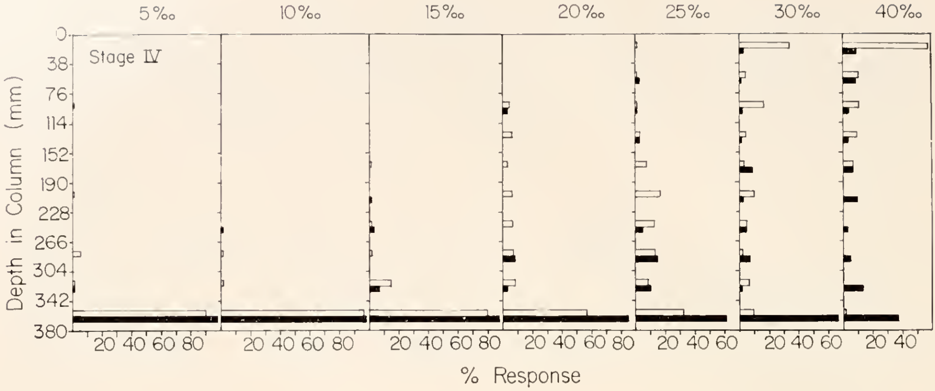

FIGURE 5. Stage IV: percentage distribution according to depth upon stimulation by 0.15 Wm^{-2} 500-nm light directed from above or in total darkness when acclimated to 20‰ and exposed to salinities between 5 and 40‰. The figure is plotted as described for Figure 4. Sample size for each condition was 60.

The percentage of Stage I zoeae positioned on the bottom of the column upon exposure to various salinities when irradiated from below is given in Table II. Due to an apparent positive phototaxis, 87% of the larvae are located on the bottom in 20‰ sea water (acclimation salinity). This percentage declines upon exposure to lower salinities as larvae move up in the water column. This ascent must be due to a negative phototaxis, since in darkness larvae descend at these salinities (Fig. 4). In 25, 30, and 40‰ (Table II), more than 90% of the larvae occur at the bottom. This indicates that at these salinities a positive phototaxis is the dominant response, as a negative geotaxis occurs in darkness (Fig. 4). In addiion, this is consistent with horizontal testing at 40‰ (Fig. 1C), in which the levels of positive phototaxis at higher light intensities are significantly greater than those at 20‰ (Fig. 1B). Thus, for Stage I zoeae, the dominant behavioral response upon exposure to low salinities is a negative phototaxis, while at higher salinities it is a positive phototaxis.

TABLE II

Percentage of larvae in bottom 4 cm of vertical column upon stimulation by 0.15 Wm^{-2} 500-nm light directed from below when exposed to salinities between 5 and 40‰. Acclimation salinity was 20‰; sample size for each salinity condition was 60 individuals.

Salinity (‰)	Percentage of larvae on bottom	
	Stage I	Stage IV
5	40	85
10	26	82
15	58	67
20	87	95
25	92	97
30	98	100
40	97	100

Exposure of Stage IV zoeae to salinities less than 20‰ (Fig. 5) results in a downward movement in both darkness and with illumination from above. The distributions in light and darkness are not significantly different. At higher salinities of 30 and 40‰, a pronounced upward movement occurs only in the light.

The negative phototaxis present at 5, 10, and 15‰ (Fig. 2A) contributes little to the vertical distribution of Stage IV zoeae (Fig. 5), since upon irradiation from below most of the larvae are found on the bottom of the column (Table II). In contrast, greater than 97% of the larvae exposed to salinities above the acclimation salinity are located on the bottom (Table II). Therefore, the dominant response by Stage IV zoeae upon exposure to low salinities is a positive geotaxis, while at higher salinities an increased level of positive phototaxis accounts for the upward movement of larvae when irradiated from above.

Anesthetized larvae sink in both high and low salinity sea water (Table III). Therefore, those larvae found up in the water column must be maintaining their position by active swimming. The larger Stage IV zoeae exhibit mean sinking rates approximately three times greater than those for Stage I. In general, sinking rates for a given larval stage increase with decreasing salinity, due to the associated decrease in density of the sea water.

Descent rates of unanesthetized Stage I and IV zoeae in 5, 10, and 15‰ in sea water with light directed from above are generally significantly greater than passive sinking rates, indicating that the descent involves active downward swimming (Table III). The exception to this is Stage IV zoeae in 15‰, where even though the mean velocity of unaesthetized larvae increased 15% over that for passive sinking, no significant difference between mean rates was obtained due to high variance. Although only mean speeds are presented in Table III, the distribution of descent speeds shown by unanesthetized larvae indicates that some larvae at each lower salinity descend by passive sinking. Furthermore, while rates of both unanesthetized Stage I and IV zoeae in 10‰ were greater than those in 15‰, possibly due to the greater salinity stimulus, rates in 5‰ were less than

TABLE III

Descent rates (cm/sec) by anesthetized and unanesthetized (normal larvae) upon sudden exposure to different salinities. Larvae were acclimated to 20‰. Velocities were measured in the vertical plane at room temperature (23–25° C). Normal larvae were irradiated from above with 500-nm light of 0.15 $W m^{-2}$ *intensity. The mean* ($\bar{x}$)*, standard deviation (s.d.) and sample size (N) are shown. The asterisk indicates that mean sinking rate for normal larvae at a particular stage and salinity is significantly greater than the mean rate for anesthetized larvae in the same salinity.*

Salinity (‰)	Stage I						Stage IV					
	Normal			Anesthetized			Normal			Anesthetized		
	$\bar{x}$	s.d.	N	$\bar{x}$	s.d.	N	$\bar{x}$	s.d.	N	$\bar{x}$	s.d.	N
5	0.46*	0.16	24	0.30	0.04	27	1.32*	0.70	23	0.98	0.09	21
10	0.66*	0.21	23	0.29	0.02	25	1.93*	0.89	23	0.81	0.10	23
15	0.54*	0.44	24	0.33	0.05	23	1.06	0.70	24	0.92	0.07	25
20	—	—	—	0.31	0.04	23	—	—	—	0.78	0.08	28
40	—	—	—	0.21	0.02	25	—	—	—	0.60	0.01	28

at 10‰. This may be caused by the shock experienced by the larvae when subjected to a 15‰ salinity decrease, rather than an active swimming response.

DISCUSSION

Phototaxis by larvae of *Rhithropanopeus harrisii* is modified by a change in salinity. Upon exposure to lower salinities at light intensities above 10^{-2} Wm^{-2}, the positive response is suppressed while the level of the negative response increases. These changes in responsiveness are more pronounced in Stage I than Stage IV zoeae. A similar reversal in the sign of phototaxis from positive to negative upon exposure to low salinity sea water has been observed in larvae of the grass shrimp *Palaemonetes* (Lyon, 1906) and the barnacle *Balanus amphitrite* (Edmondson and Ingram, 1939).

A decrease in salinity of only 1.1‰ from the acclimation salinity will induce this reversal in the sign of phototaxis in Stage I zoeae acclimated to 20‰. The minimum salinity decrease necessary for inducing this behavior in Stage IV zoeae and in larvae acclimated to 10‰ is comparable (within 1.0‰). Since these values are similar, the salinity change threshold apparently does not depend upon acclimation salinity or developmental age. Instead, larvae are responding to the salinity decrease from that particular acclimation salinity and not to an absolute salinity.

These results indicate a high sensitivity to salinity in a euryhaline brachyuran larva (Costlow *et al.,* 1966), which compares to that by larvae of the stenohaline polychaete *Polydora pulchra.* For this polychaete, a salinity decrease of 1.5‰ from that in its natural environment (34‰) induces a negative phototaxis (Ranade, 1957). According to Thorson (1964), larvae of intertidal species require a much greater salinity decrease to reverse the sign of phototaxis than those from subtidal species. Indeed, this is true for several species, such as larvae of *Balanus amphitrite,* in which a 50% dilution of seawater is necessary to initiate a negative phototaxis (Edmondson and Ingram, 1939). This generalization, however, is questionable, since *R. harrisii* is a low intertidal species (Bousfield, 1955; Pinschmidt, 1963; Smith, 1967).

The negative phototaxis that is induced in larvae of *R. harrisii* by lowering the salinity is clearly a short-term response. A positive phototaxis is totally recovered in 5.5 minutes, with a 50% return in 1.5–3.2 minutes. Whether exposed to a 1.3‰ or a 15‰ salinity decrease, these times are similar. Thus, the return of a positive phototaxis seems independent of both the developmental stage and the amount of salinity change beyond the threshold value. Similar times have been observed for nauplii of *B. amphitrite,* which upon exposure to 50% sea water regain a positive phototaxis in ten minutes (Edmonson and Ingram, 1939). The short-term recovery of the positive phototaxis upon a sudden change in salinity may possibly be a general characteristic of pelagic larvae.

Stage IV zoeae are positioned lower in the experimental water column than Stage I in darkness and under overhead light at the acclimation salinity. This general pattern has been predicted (Ott and Forward, 1976) and observed in the field (Sandifer, 1975) for larvae of *R. harrisii.* A deeper net distribution of later stages has also been noted for estuarine larvae of the crabs *Leptodius floridanus* and *Panopeus herbstii* (Sulkin, 1973; 1975), the shrimps *Penaeus duorarum* (Hughes, 1969) and *Macrobarchium acanthurus* (Hughes and Richard, 1973), and the barnacle *Balanus improvisus* (Bousfield, 1955). The mechanism underlying these distribution patterns is unknown.

Previous laboratory studies indicate that downward movement is induced in zooplankton upon exposure to lower salinity sea water. This has been observed in 17 copepod species (Grindley, 1964; Harder, 1968; Lance, 1962), the branchiopod *Artemia salina* as well as nauplii of the cirripeds *Pollicipes polymerus* and *Balanus tintinnabulum* (Harder, 1968), and larvae of the decapods *Macrobrachium acanthurus* (Hughes and Richard, 1973), *Porcellana longicornis* (Lance, 1962), *Paguris longicarpus* (Roberts, 1971), and *Homarus americanus* (Scarratt and Raine, 1967). Generally two mechanisms of response have been observed: directed downward swimming and passive sinking. Whether the downward swimming is due to a positive geotaxis and/or negative phototaxis had not been determined. In the present study, the components involved have been identified.

Although the vertical distributions of both Stage I and IV zoeae of *R. harrisii* in light and darkness upon exposure to salinities below the acclimation salinity are similar, the behavioral response mechanisms underlying their distribution are not uniform. The descent by both zoeal stages in darkness is due to a postive geotaxis generally involving active downward swimming. Although a positive geotaxis can contribute to the descent under overhead light, the dominant behavioral response for Stage I zoeae is a negative phototaxis, since the larvae ascend upon irradiation from below. In contrast this ascent is much weaker for Stage IV zoeae, so a negative phototaxis contributes little to downward movement under overhead light, and the positive geotaxis is the main behavioral response.

The ascent by larvae upon an increase in salinity does not result from floating due to the increased density of water, since anesthetized larvae sink in the higher salinities. Stage I zoeae are positioned high in the water column due to a negative geotaxis in darkness. Again, the geotaxis can contribute to movements under overhead light, but the primary behavioral response is a positive phototaxis, since the larvae descend upon irradiation from below. In contrast, Stage IV zoeae only show a pronounced ascent due to a positive phototaxis when irradiated from above. In darkness, a very weak upward movement is observed, which implies that this zoeal stage will only ascend upon exposure to a salinity increase during the day.

In coastal plain estuaries usually a moderate amount of vertical stratification by salinity exists, in which an upper layer of low salinity sea water is separated from a lower layer of high salinity sea water by a region of water characterized by a salinity gradient (Pritchard, 1967). Pinschmidt's (1963) study in the Newport River estuary (North Carolina) supports this generalization, since he demonstrated that at the two stations where *R. harrisii* larvae were most abundant, mean salinity differences between surface and bottom water were 2.3‰ during high tide and 1.5‰ for low tide. A more recent unpublished study by Thomas Cronin (Duke University Marine Laboratory) indicates that in areas of this estuary where the larvae occur, salinity differnces between the surface and bottom can be as high as 9‰, but the average is around 5‰. Furthermore, in the Neuse River estuary (North Carolina), monthly salinity readings over the course of three summers from two stations in the vicinity of the collecting site for the present study indicate that this region is also stratified, with a mean salinity differential between surface and bottom of 1.8‰ (Hobbie and Smith, 1975). Thus salinity changes throughout the water column do exist in areas where *R. harrisii* larvae occur and are sufficient to evoke behavioral responses.

The predicted general behavior of larvae confronted with salinity variations is as follows. Larvae entering the upper layer of reduced salinity sea water will experience a decrease in salinity and consequently respond by a downward movement due to a positive geotaxis and/or negative phototaxis and therefore descend into sea water of the original salinity. A decrease of only 1–2‰ will induce the negative photoaxis. While a positive geotaxis is clearly induced by a 5‰ change, it seems probable that the thresholds for this response are similar to those for the negative photoaxis. As larvae regain normal phototactic responsiveness at a new salinity in 5.5 minutes, a salinity decrease would have to be experienced over a very short time span.

Although the return to a positive phototaxis occurs quickly, there is sufficient time for larvae to negotiate a substantial portion of the water column. Mean descent rates of larvae acclimated to 20‰ and experiencing a 5–10‰ salinity decrease in the presence of light are 0.6 cm/sec for Stage I and 1.5 cm/sec for Stage IV. Within the span of time allowing for a 50% to full recovery of the positive phototaxis, it is calculated that Stage I zoeae can transverse a vertical distance of 1–2 m and Stage IV 1.4–5 m. In the region of two estuaries where larvae are abundant, mean depths at low tide are 3.5–5 m (Bousfield, 1955; Pinschmidt, 1963). The short-term recovery indicates that if a larva cannot lose the negative response by escaping from the salinity region which induced it, the response will be lost over time, thereby allowing the animal to acclimatize to a new salinity and resume normal behavior.

Alternately, larvae could move down into sea water of a higher salinity than that to which they are acclimatized. In this case, they swim upwards due to a positive phototaxis and/or a negative geotaxis. Therefore, these behavioral responses to increases and decreases in salinity can act as a negative feedback system to keep larvae within the region of acclimatization salinity water.

A limitation of the present study is that only sudden changes in salinity were considered. At present nothing is known about rates of salinity change, which are necessary to initiate these behavioral responses. In addition, the physiology of these responses is relatively unstudied. For example, the site of salinity detection is not known. Roberts (1971) demonstrated that it is not on the uropods or antennae of larvae of *Paguris longicarpus,* as amputated animals showed identical salinity responses as nonamputees. Also, the mechanism whereby a salinity decrease reverses the sign of phototaxis, and even the site of gravity reception for geotaxis, are unknown. As zoeae possess no functional statocyst (Prentiss, 1901), perhaps the receptors are located in the numerous spines covering the carapace (Foxon, 1934). The need for suitable studies on these aspects of larval physiology is evident.

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SUMMARY

1. Experiments were conducted to determine the effect of salinity on phototaxis and geotaxis by Stages I and IV zoeae of the crab, *Rhithropanopeus harrisii.*

2. Larvae were exposed to sudden salinity changes and stimulated with various intensities of 500-nm light in the horizontal plane. Although the pattern of phototaxis of larvae exposed to 40‰ was unchanged from that at 20‰ (acclimation salinity), the level of positive phototaxis to higher intensities was significantly greater and the level of negative phototaxis to low intensities significantly lower at 40‰. Exposure to low salinity sea water (5‰) generally reverses the sign of phototaxis, since a significantly higher level of negative phototaxis and lower level of positive phototaxis occurs at light intensities above 10^{-2} Wm^{-2}.

3. The minimum amount of salinity decrease from the acclimation salinity that induces a reversal in phototactic sign from positive to negative phototaxis at 0.19 Wm^{-2} ranges from 1 to 2‰, and appears to be independent of acclimation salinity and developmental stage. Total recovery of a positive phototaxis occurs in approximately 5.5 minutes for both zoeal stages, with a 50% return apparent in 1.5–3.2 minutes.

4. Larvae stimulated from above with light of 0.15 Wm^{-2} or maintained in darkness in a vertical column exhibited salinity-dependent as well as age-dependent vertical distributions. At each of the seven test salinities (from 5 to 40‰), Stage IV zoeae maintained a lower position in the column than Stage I.

5. Stage I had similar vertical distributions in darkness and overhead light. At salinities below the acclimation salinity larvae moved downward due to a positive geotaxis and negative phototaxis. Upon exposure to higher salinities, an upward movement due to a negative geotaxis and positive phototaxis occurs. Phototaxis, however, is the dominant behavioral response in light.

6. Stage IV zoeae migrate down in overhead light and darkness upon a decrease in salinity. The dominant behavioral response is a positive geotaxis. With an increase in salinity, as ascent only occurs under overhead light, indicating movement results from a positive phototaxis.

7. Anesthetized larvae sink in both high and low salinity water. Thus, the ascent in high salinities does not result from floating due to the increased density of the water. A comparison of descent rates by anesthetized and unanesthetized larvae in 0.15 Wm^{-2} light directed from above and in low salinity water indicates that the normal descent results primarily from active downward swimming, although some larvae exhibit passive sinking.

8. These behavioral responses to increases and decreases in salinity can act as a negative feedback system to keep larvae within the region of acclimatization salinity water in the vertical water column.

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LOCOMOTION AND PROPAGATED SKIN IMPULSES IN SALPS (TUNICATA: THALIACEA)

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With the notable exception of the work of M. Fedele, whose major papers on salps in the period 1923–1933 are cited below, few serious attempts have been made to analyze the behavior of salps. Yet, being large transparent animals with clearly visible and well-defined muscle bands and nerves, with compact brains and interesting behavior, they and the doliolids are potentially the most promising of the tunicates for neurophysiological investigations. Because of their scarcity in most coastal regions, salps have been little studied. The present report is the first electrophysiological investigation of salp behavior and is correspondingly incomplete or tentative in many respects. The mechanism of locomotion has been studied using techniques similar to those adopted in previous studies of tunicate behavior. These include investigations of the locomotory control mechanisms in the larvacean *Oikopleura* (Bone and Mackie, 1975) and in the tadpole larva of an ascidian, *Dendrodoa* (Mackie and Bone, 1976). In both these cases, and now in salps, evidence has been obtained for the existence of "neuroid" conducting epithelia (Mackie, 1970) and for their role as sensory pathways in responses involving changes in locomotory activity.

MATERIALS AND METHODS

The salps used in this study were caught in plankton nets or scooped from the water in glass bottles in the bay of Villefranche-sur-Mer, France, during the period December 1973 to March 1974. They were kept in circulating seawater aquaria at the Station Zoologique and were used for experiments soon after capture.

Taxonomic and biological accounts of the species used (*Thalia democratica, Salpa fusiformis, Salpa maxima, Ihlea punctata,* and *Pegea confoederata*) are given by Branconnot (1973). Ihle (1933, 1958) provides indispensible background information on salp morphology and relationships.

Formalin-fixed and fresh tissues were examined by Nomarski and phase contrast microscopy. Material was fixed for electron microscopy in 5% cacodylate-buffered glutaraldehyde in sea water, followed by postfixation in 1% osmium tetroxide. Sections were stained with uranyl acetate and lead citrate prior to examination with a Philips EM-200.

Fine polyethylene suction electrodes were used for recording externally from the muscle bands and conducting epithelia. Signals were amplified and displayed on a Grass Polygraph and simultaneously on a Tektronix storage oscilloscope. A Grass stimulator was used for electrical stimulation. Intracellular recordings were made with glass micropipettes filled with 3 M KCl having resistances in the range

30–50 megohms, in conjunction with a Medistor A35 electrometer amplifier and an FET amplifier having current injection bridge circuitry.

RESULTS

Swimming patterns and muscle physiology

Locomotion in salps is brought about by rhythmic contractions of the muscle bands which lie in the body wall, acting in conjunction with the muscles of the inhalent and exhalent valves. Locomotion is normally in the forward direction, water being expelled through the posterior, exhalent valve. In the lab, recently collected salps swim most of the time but show quiescent interludes. In nature they probably swim nearly all the time and must do so in order to feed (Madin, 1974). There is no evidence that salps can control the angle at which they swim or that locomotion is directional, in the sense that they show behavioral taxes. However Fedele (1923, 1933b) showed that it was possible to alter the swimming pattern by appropriate stimulation. Stimulation of the hinder regions and exhalent valves causes accelerated forward swimming. Stimulation of the front region and inhalent valves causes a defensive response, which consists of an alteration of the normal sequence of muscle contractions: the exhalent valve shuts first (instead of the inhalent), followed by contraction of the body wall muscles. Thus, water is expelled from the inhalent valve. A similar response is seen when the inside of the pharynx is stimulated (Fedele, 1933a, c). These movements would presumably serve to prevent unsuitable objects from entering or becoming lodged in the pharynx, as does the squirting of a sessile ascidian, but they also automatically interrupt locomotion. Fedele (1923) states that these defensive movements can be elicited by photic, thermal, chemical or tactile stimuli.

Mature blastozooids of *S. fusiformis* exhibit a swimming rhythm of 0.5–0.8 beats per second. Suction electrodes attached to the muscle bands show that each contraction is correlated with a burst of muscle potentials (Fig. 1A). Resolution of these potentials, which rarely exceed 200 μV, falls off as the electrode is moved away from the muscle band, but an electromyogram can be picked up from any part of the animal even, in small specimens, from the surface of the test.

In forward locomotion the muscles which operate the inhalent valve fire about 40 msec before the main muscle bands and the muscles of the exhalent valve (Fig. 1B). Recordings from different muscle bands in the body wall show close synchrony in the onset and conclusion of contraction bursts both during forward (Fig. 1C) and reverse (Fig. 1D) locomotion, and it is evident that the main musculature fires more or less as a unit.

Gentle tactile stimulation of the inhalent valve (on its inner surface in Fig. 2A) causes a break in the rhythm of forward swimming, while stimulation of the exhalent valve causes accelerated forward swimming (Fig. 2B). Fedele's defensive response is exhibited in varying degrees according to the strength of stimulation. With weak stimulation, it may be a barely preceptible hesitation in the rhythm. With strong stimulation, prolonged inhibition or reverse locomotion takes place. These responses are accompanied by conducted epithelial potentials (skin pulses) which will be described below.

The reverse beat is a more powerful contraction than the forward and con-

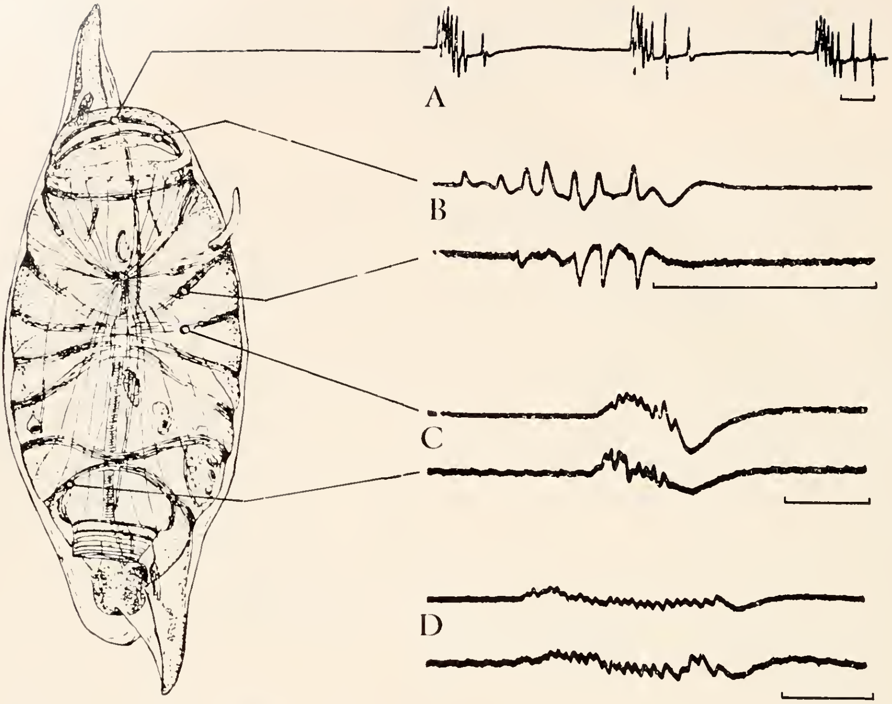

FIGURE 1. *Salpa fusiformis*, dorsal view of a blastozooid (from Fedele, 1933b) with extracellular recordings from the muscle bands (scales are 100 msec): A. forward swimming, series of contraction bursts from a muscle of the inhalent valve; B forward swimming, a single burst recorded simultaneously from the inhalent valve and from body wall muscle band I; C. forward swimming, a burst recorded from right band II and left band VI; and D as for C, but reverse swimming response.

sists of a longer burst of potentials (Fig. 1D). The beat frequency is also lower. The reversal of the firing sequence is illustrated in Figure 3. After a period of reverse locomotion, forward swimming is resumed spontaneously, sometimes after a very brief delay, sometimes after a longer interval. A residual inhibitory effect is

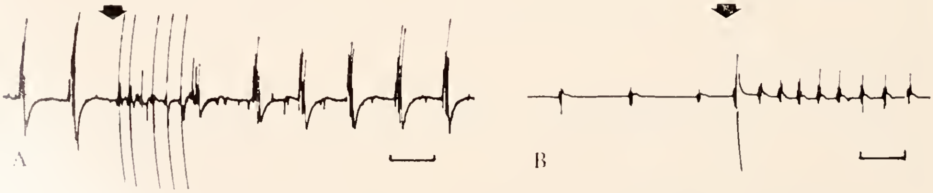

FIGURE 2. Alteration of the swimming rhythm in *S. fusiformis* (scales are 1 sec): A, blastozooid—a stimulus (arrow) elicits a burst of five anterior skin pulses and also causes a brief interruption of swimming, as seen in the retardation of swimming bursts and their attenuation; and B, young oozoid—a stimulus at the rear end (arrow) evokes a single outer skin pulse and accelerated swimming.

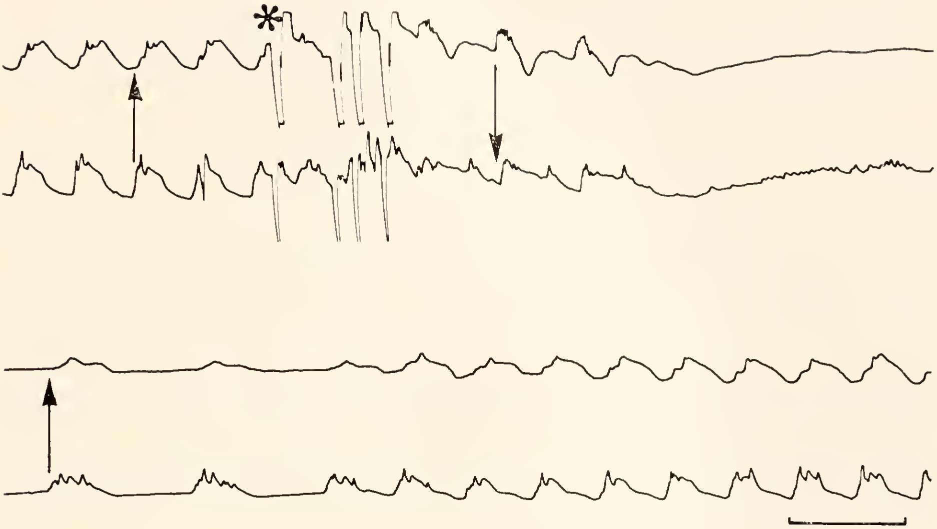

FIGURE 3. *S. fusiformis:* reversal of swimming. In each pair of records, the upper electrode was attached to the exhalent valve, the lower to the inhalent. Tactile stimulation of the inhalent valve (at asterisk) evoked a series of four outer skin pulses and the direction of swimming changed from forward (lower channel leading, arrow up) to reverse (arrow down). Reverse swimming then gave place to a period of complete inhibition of swimming. Eight seconds are omitted between the two pairs of records during which slow forward swimming (arrow up) was established, subsequently giving way abruptly to fast forward swimming. (Scale is 1 sec).

evident in the weakness of the first forward swimming beats after a period of quiescence, and the frequency may be abnormally low during this phase. This slow, "inhibited" forward swimming may change gradually to the normal pattern, but sometimes an abrupt change of pace is seen (Fig. 3).

The locomotory rhythm is markedly affected by changes in light intensity. Salps which are swimming rhythmically accelerate following a reduction in light intensity, and those which are quiescent can often be induced to start. An increase in intensity slows the rhythm or causes locomotory arrest. The significance of these reactions in the natural behavior of salps in the sea is uncertain. It is unlikely that vertical distribution is influenced by diurnal changes in light intensity (C. Apstein, cited by Ihle, 1958). The light OFF response might be a predator avoidance reaction ("shadow reflex") of the type described in some hydromedusae (Yoshida and Ohtsu, 1973).

The muscle bands in *S. fusiformis* are composed of parallel elongated fibers, flattened in cross section and showing cross striations. They differ little from those of other salps such as *Iasis* and *Thalia,* whose ultrastructure and innervation have been described recently (Bone and Ryan, 1973). There are several neuromuscular junctions on each fiber, derived from axons passing out from the central nervous system in different nerve bundles. A single axon may innervate several muscle fibers; as Fedele (1925) showed, endplates may be intercalary.

To prevent swimming, it is only necessary to sever the nerves between the

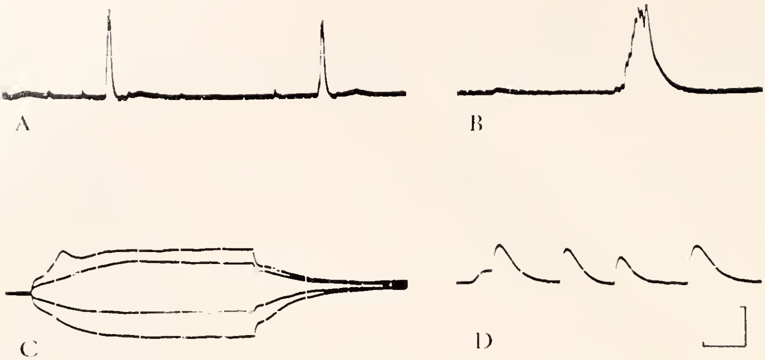

FIGURE 4. Intracellular records from salp muscle bands (A-C, *S. fusiformis;* D, *Ihlea punctata*)*:* A, contraction bursts recorded in an intact, swimming animal (scales are 500 msec, 10 mV); B, a burst from the same preparation on expanded time scale (100 msec, 10 mV); C, denervated fiber injected with hyper- and depolarizing current pulses of 2×10^{-8} and 4×10^{-8} A. Note rectification of the response to the stronger depolarizing current pulse (scale is 20 msec, 20 mV); and D, denervated, fibrillating fiber showing junctional potentials recorded near a neuromuscular junction (scale 50 msec, 10 mV).

brain and the muscles. This was done in experiments which required placement of two microelectrodes in the same fiber simultaneously. The muscles can be made to contract by stimulating their motor nerves, but the nerves are too small for this to be done conveniently.

Intracellular recordings from muscle fibers in *Salpa fusiformis* and *Ihlea punctata* showed negative resting potentials in the range 50–55 mV. During spontaneous foward swimming, bursts of potentials summing to a maximum amplitude of 25 mV were recorded (Fig. 4A, B). These potentials correspond to the potentials recorded extracellularly, shown in Figure 1. The amplitude of contraction presumably depends on the duration of the burst and on the number and frequency relationships of the potentials, which will determine the level of depolarization, but this needs to be verified directly. During partial photic inhibition of swimming the amplitude of the summed potentials may drop by more than 50%, which visibily results in feeble contractions.

We regard these potentials as neuromuscular junctional potentials (JPs). Their numbers and frequency relationships probably reflect the pattern of motor nerve impulses arriving at the junction. Recordings from presumed motor neurons in the isolated brain show similar patterns of impulses. Scattered JPs or small bursts of these potentials are sometimes seen in the intervals between the swimming contractions (Fig. 4A, B), but they cause no detectable shortening of the fiber. Denervated fibers are usually electrically silent at first, but start to show increased JP activity after a few minutes and eventually start to fibrillate. These changes are exacerbated by operations in the region of the muscle which involve opening up the haemocoel.

Salp muscle fibers show active membrane responses to depolarizing currents injected through a microelectrode (Fig. 4C). These responses are graded according to the amount of current passed. The largest responses induced had amplitudes of 20–25 mV. Responses have been seen with as little as 10 nAmp current.

Current pulses injected into one fiber induced no measurable voltage changes in the fibers on either side. This lack of lateral coupling is in accordance with the absence of gap junctions between adjacent fibers reported by Bone and Ryan (1973).

While it is hard to visualize neuromuscular junctions in an intact, swimming salp because of the movement and thickness of the preparation, it is not hard to see the junctions in denervated, flattened muscle preparations and to insert electrodes at known distances from neuromuscular junctions. Fibrillation potentials (Fig. 4D) recorded close to junctions appeared to be larger than the same events recorded further away; while this observation cannot yet be put on a precise quantitative basis, it serves as an indication that the junctions are still the sites of origin of the potentials recorded after denervation. Fedele (1933b) found that, in muscle bands partially isolated from the brain by nerve section, paralysis was restricted to denervated zones. This observation fits the general picture arrived at here, as it indicates that the muscle fibers are incapable of propagative electrogenesis.

There is good evidence for cholinergic transmission in tunicates (Florey, 1967), and salp neuromuscular junctions are characterized by vesicles resembling known cholinergic vesicles in other animals. The presumption of cholinergic transmission is supported by our observations on the effects of acetylcholine, curare and eserine on exposed muscle fibers; but the effects of these and other drugs need to be explored further and will be reported elsewhere.

Origin of the locomotor rhythm

Fedele (1933b) showed by surgical operations, and we have confirmed, that the swimming rhythm originates in the brain. Severing the nerves to the muscles causes paralysis. A few recordings were made from isolated brains removed from the animal and pinned out in sea water after dissecting away the covering epithelia. On a single occasion, a cell showing the characteristics of a pacemaker neuron was penetrated. This cell, from the brain of *Thalia democratica*, was impaled for 20 min, during which it fired regularly 1.5–1.7 times per second (Fig. 5A, B). Spike threshold lay at −55 mV. Following the spike, the cell hyperpolarized to −65 mV, then depolarized again to a new spike threshold. There was no sign of synaptic input and, although the cell could have been driven by another cell or cells, it was assumed to be endogenously rhythmic. Spike peak lay at −40 mV, which suggests that the electrode was in the cell soma, which was passively invaded by spikes initiated at some distance down the axon (*c.f.*, Hoyle and Burrows, 1973). Hyperpolarizing the cell slowed the rhythm and depolarizing it accelerated it (Fig. 5C, D). Folowing the current pulse, rebound excitation and inhibition effects were evident.

A second type of cell was identified in several separate recordings from brains of *Salpa fusiformis* (Fig. 5E, F). The electrode was situated in the superficial layer of the brain near the posterior end, the region in which large motoneuron somata are located (Fedele, 1933b). The low resting potential (−25 mV) and small size of the transient potentials are incompatible with a normal, stable intracellular placement. Presumably, these are proximity recordings or represent partial penetrations. The rhythm in this case (0.5/sec) lay within the normal

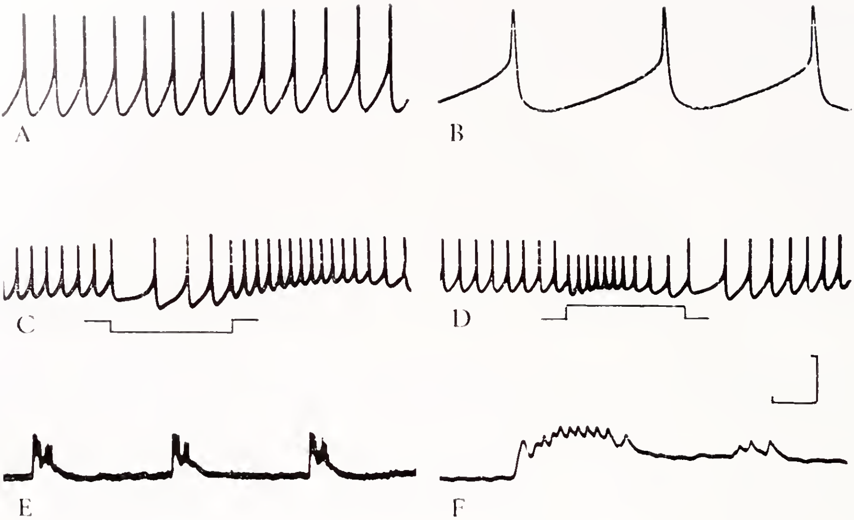

FIGURE 5. Recordings from isolated brains in *Thalia democratica* (A-D) and *Salpa fusiformis* (E, F). The former are from a pacemaker neuron and the latter from a presumed locomotory motoneuron, as explained further in the text. Scales are: A, 1 sec, 10 mV; B, 200 msec, 10 mV; C, D, 2 sec, 20 mV; E, 1 sec, 10 mV, and F, 50 msec, 10 mV.

range for the locomotory rhythm of this species and size of salp. As in the intact animal, the rhythm was affected by changes in light intensity (Fig. 6). This would be expected, since the brain incorporates ocelli. The pattern of events comprising the bursts resembles the pattern of JPs recorded from muscle cells both in number and frequency relationships. There can be little doubt that cells showing these impulse patterns are the motor neurons which control swimming.

The salp brain obviously merits further study. The circuitry is probably fairly simple. Fedele (1933b) found small "commissural" neurons in the outer layer (rind) which, he suggested, were responsible for synchronizing the activity of the mononeurons. He found that destruction of the central core of the ganglion failed to interfere with the rhythm, but that thereafter the rhythm was no longer modifiable by sensory input. Damage to the rind interfered directly with the

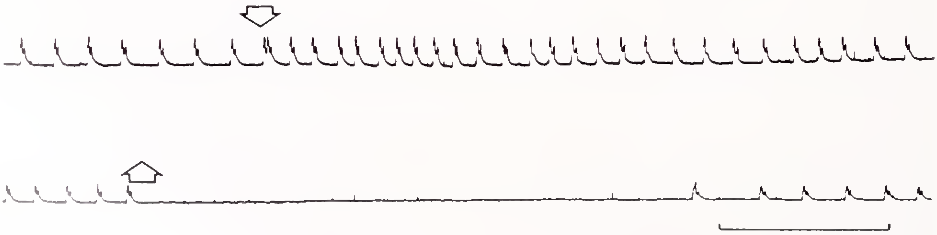

FIGURE 6. Microelectrode recording from presumed motor neuron in the brain of *S. fusiformis*. At light-OFF (arrow down) the rhythm is accelerated for about 10 seconds. At ON (arrow up) swimming is inhibited for 33 seconds. Scale is 10 sec.

rhythm. We also have found that a needle can be thrust repeatedly through the center of the brain without destroying the rhythm. It appears that the rhythm is generated by neurons with somata in the rind, while the core is the site of synaptic input from afferent neurons.

Skin pulse system

The term skin pulse (SP) refers to an impulse generated and conducted by epithelial cells without the intervention of nerves. However SPs can excite neurons. In *Oikopleura* excitation evoked by touching the skin is transmitted by skin cells as SPs, enters neurons and is conducted to the caudal ganglion, where it causes a change in the locomotory rhythm (Bone and Mackie, 1975). Conducting epithelia are well-developed in the Salpidae. The following account refers to *S. fusiformis*, where the excitable epithelia have been localized to three regions. The functional significance of this zonation is still unclear.

The main zone consists of the outer, ectodermal epithelium which underlies the test. Signals recorded from this epithelium (Figs. 2B, 8, 9, 11) are referred to as outer skin pulses (OSPs).

A second zone (in fact, a pair of similar zones) consists of the endodermal epithelium covering large areas of the pharynx and the walls of the gill bar. The area to the left of the endostyle including the left wall of the gill bar forms one self-contained conducting field, and the equivalent areas on the right form another (Figs. 7, 8). The pharyngeal wall is excitable only in its ventral and lower lateral regions, up to a line approximately level with the bottoms of the muscle bands. Sensitivity disappears rapidly dorsal to this line except in the gill bar, which is sensitive up to its dorsal attachment. Reference will be made to left and right inner skin pulses (LISPs, RISPs).

A third zone is the area of inner epithelium lying between the peripharyngeal bands and the border of the inhalent siphon (Fig. 7). Impulses recorded in this zone may be termed anterior skin pulses (ASPs), examples of which are shown in Figure 2A. The comparable area to the rear of the animal, posterior to the ventral attachment of the gill bar is not excitable.

Delineation of the excitable zones is complicated by the fact that skin impulses are recorded extracellularly as large events, often exceeding 1 mV and can consequently be picked up by electrodes quite far from the active site. Even LISPs and RISPs, which are relatively small events (less than 0.5 mV), can sometimes be picked up by a sensitive electrode in each other's territories (Fig. 8). The excitable epithelia enclose the haemocoel and electrotonic current flow through the blood is probably responsible for signal pickup in distant regions. OSPs can be recorded on the pharyngeal side, although their wave forms may be inverted or distorted here compared with recordings from the outer skin itself.

The location of the inner and anterior skin pulses in their respective zones has been verified by careful stimulation with fine glass needles. There is both indirect and direct evidence that OSPs are conducted by the less accessible outer epithelium. They are recorded at higher amplitudes and with fewer distortions from the outer epithelium than from the inner. They are conducted into the anterior and posterior prolongations of *S. fusiformis* blastozooids, where there is no inner epithelium. Direct proof of their location comes from microelectrode recordings from the outer

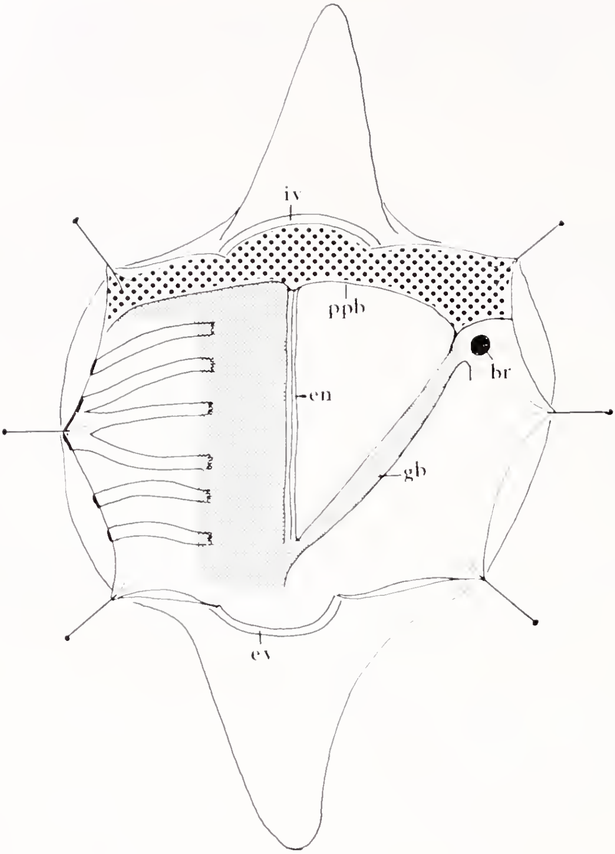

FIGURE 7. *S. fusiformis*, blastozooid: territories of anterior skin pulses (spots) and left inner skin pulses (stipple). The salp has been opened up from the dorsal side and pinned out with the gill bar to the right. Muscles are shown only on left. The right inner skin pulse zone is the mirror image of the left (br represents brain; en, endostyle; ev, exhalent valve; gb, gill bar; iv, inhalent valve; ppb, peripharyngeal band).

epithelium itself (Fig. 9). Here, a window was cut in the pharyngeal wall and a microelectrode was passed through it and inserted into the outer epithelium. Spikes recorded intracellularly from the epithelium correlated perfectly with events identified as OSPs on other grounds.

The epithelia are extremely thin. In electron micrographs of a young *S. fusiformis* blastozooid, the outer epithelium was found to be 0.5–1.4 μ thick, the inner 0.3–0.8 μ. The intracellular recordings were made from an older specimen which may have had thicker epithelia, but the tissues would still have been relatively thin, certainly less than 5 μ thick. Only three penetrations were made, all in the outer layer. The low resting potential (35 mV) and nonovershooting spike are indicative of cell injury. Penetration was only momentary. While these records serve to confirm the location of OSPs in the outer skin, the tissue is clearly unsuitable for prolonged microelectrode work, and a thicker conducting epithelium must be sought if investigations are to be carried out on the electroionic characteristics of salp conducting epithelia. The ectoderm of the young stolon might be such a layer.

In conducting epithelia in amphibian tadpoles (Roberts, 1975) and hydrozoan coelenterates (Mackie, 1976) the cells are electrically coupled, and cell to cell transmission occurs by direct current flow, presumably through gap junctions, which have been located in the appropriate regions. A similar principle can be assumed to apply for tunicate conducting epithelia. Gap junctions (Fig. 9C) are seen connecting the cells of both inner and outer skin layers. The junctional region between adjacent cells is typically convoluted, and a *zonula occludens* occurs at the outer edge.

Brain removal and other operations which involve nervous or muscular lesions have no effect on the SP systems except insofar as they may damage, and so excite, the epithelia. The SP systems are not photosensitive. They are probably not spontaneously active but, being readily excited by damaging stimuli, are often exhibited in pinned preparations, where they may fire singly or in long bursts without apparent cause. In some cases, swimming movements, which would involve friction of the epithelia against pins or electrodes, appear to set off SP bursts. When a blastozooid undergoes autotomy from its neighbors in the chain, intensive OSP activity is recorded, probably due to damage of the outer epithelium at the adhesion plaques.

From the few, imperfect intracellular records obtained, OSPs are apparently conventional action potentials of approximately 10 msec duration (Fig. 9B), resembling epithelial spikes recorded in coelenterates (Mackie, 1976; Schwab, University of California, Irvine, personal communication) more closely than they do the action potentials recorded from larval amphibians (Roberts, 1975) and ascidian tadpoles (Mackie and Bone, 1976). Conduction velocity in the outer skin was measured at 17 cm/sec at 18° C in one preparation. In the same preparation ISPs were conducted along the gill at 8.5 cm/sec. The absolute refractory period for OSPs was measured at 7 msec in one example. OSPs can fire at up to 5/sec in the early stages of bursts.

Epithelial excitability is established early in embryonic development both in

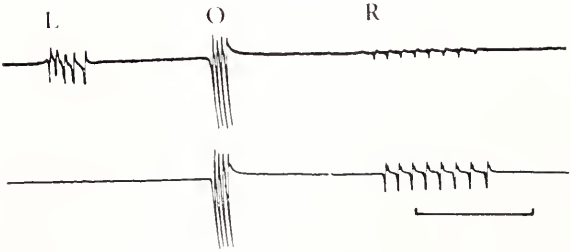

FIGURE 8. Comparison of OSPs, LISPs and RISPs recorded consecutively from the pharyngeal surface (*S. fusiformis*). The upper electrode was on the left of the endostyle, the lower on its right. Scratching the left side (at L) evoked LISPs, touching the right (R) evoked RISPs. OSPs were evoked by touching the outer surface of the salp, and are picked up electrotonically. Scale is 1 sec.

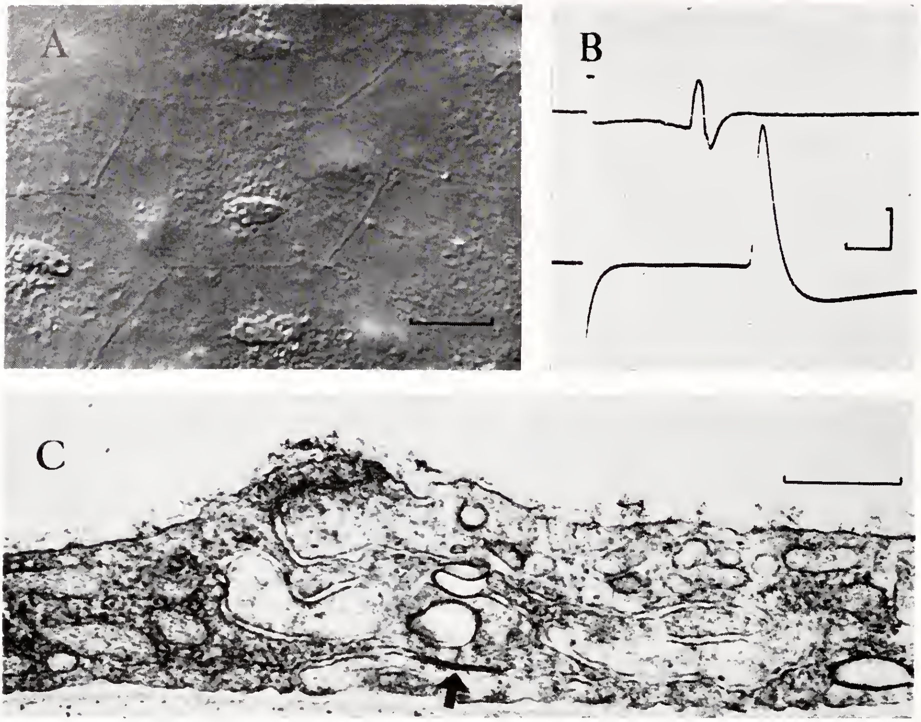

FIGURE 9. A shows epidermis of *S. fusiformis*, by Nomarski interference contrast microscopy) (scale is 20 μ). B shows a skin pulse recorded from this layer extracellularly (upper trace) and intracellularly (lower). The extracellular electrode was nearest to the stimulating site. The delay between the appearance of the signal on the two channels represents conduction time in the epithelium (scale is 10 msec, 500 μV, extracellular and 10 mV, intracellular). C shows a section through the outer epithelium in a junction region, showing a gap junction (arrow) (scale is 250 nm).

the case of oozoids and blastozooids. OSPs have been recorded in young oozoid embryos still attached to the parent, and in stolons in the process of subdivision into blastozooid chains. The question of SP communication in salp chains is discussed further below.

Skin pulses have no effect on the heartbeat, which is myogenic. There may be a delayed effect of ISPs on the beating of the gill bar cilia, but a nerve bundle runs through the bar and it is impossible to exclude the possibility that the effect on the cilia is due directly or indirectly to nervous excitation. Fedele (1933a) notes that food collection in salps involves coordination of ciliary activity in the gill bar and esophagus with mucous secretion by the endostyle. There is a food rejection reflex which involves muscular movements of the gut, cessation of mucous secretion and dissolution of the food web. Fedele attributes control of these effectors to the visceral nervous system, but the existence of the ISP system must now be taken into account.

The most obvious functional role for OSPs and ASPs lies in the regulation of locomotion. The sorts of tactile stimulation which cause acceleration, inhibition and reversal of swimming almost always give rise to skin pulses. In Figures 2 and 3, for example, SPs are seen preceding or accompanying the change in swimming pattern, and appear to be causative of such changes. Epithelial pulses generated by touch are envisaged entering the nervous system and being relayed, as nerve impulses, to the brain. The most likely route is *via* the sensory nerve endings known to occur in the outer skin (Bone, 1959).

In a few cases an alteration was produced in swimming by delicate local stimulation which failed to elicit skin pulses. Here, direct stimulation of the sense organs may be presumed to have occurred.

If this interpretation is correct, the excitable epithelia would be functioning essentially as in *Oikopleura* (Bone and Mackie, 1975), extending the sensory field around ciliated neurosensory receptors lying within the epithelial sheets.

While it is difficult to elicit changes in locomotion without evoking skin pulses, a great deal of skin pulse activity can occur without any visible effect on locomotion. This is a common observation in pinned preparations where the stimulation due to the pins sets off frequent SP discharges. It appears likely that adaptation or fatigue occurs quickly at some step or steps along the sensory pathway. In nature, SPs are probably infrequent events which can affect swimming, but cease to do so on over-stimulation.

Coordination in salp chains

Fedele (1923) investigated the behavior of salp chains and found that, although the individual zooids have independent rhythms, all connected zooids show the same type of activity at any given time: in a resting chain, if one salp starts to swim, the others also start; if one stops, all stop; if one accelerates, so do most of the others; reverse swimming is propagated down the chain, for instance when the leading salp strikes an impassable object. For the chain, as for the individual, chemical, thermal, photic and tactile stimulation elicit these changes. If a chain is pinched in the middle, the two parts separate, the anterior half swimming forward at an accelerated speed, and the posterior going into reverse and swimming in the opposite direction.

We have little to add to Fedele's behavioral observations; so far as our own go, they confirm his. As Fedele observed, young chains are more responsive than older ones, and show reverse locomotion more readily. Some old chains will only swim forward. In chains of *Salpa maxima* whose zooids measured 1.5 cm in length no propagated responses of any kind were found, but in *S. fusiformis* and *P. confoederata* coordination was very clear. *S. fusiformis* chains swim equally well in either direction at velocities within the range 2.0–5.0 cm/sec, and all zooids swim in the same direction at any given time.

The question of how coordination is achieved has yet to be resolved. Ectodermal and endodermal continuity exists in stolons and young chains, but the endodermal connections are lost very early, and in some species the ectodermal connections become slender tubes (Fig. 10A) abutting on the adhesion plaques. There is no epithelial continuity across the plaque in older chains. Collections of ciliated sensory cells terminate at the plaques (Fedele, 1923; Bone, 1959), but nerves apparently do not cross directly from one zooid to the next. The flag organs (Bolles Lee, 1891) which are found only in aggregate salps and seem to

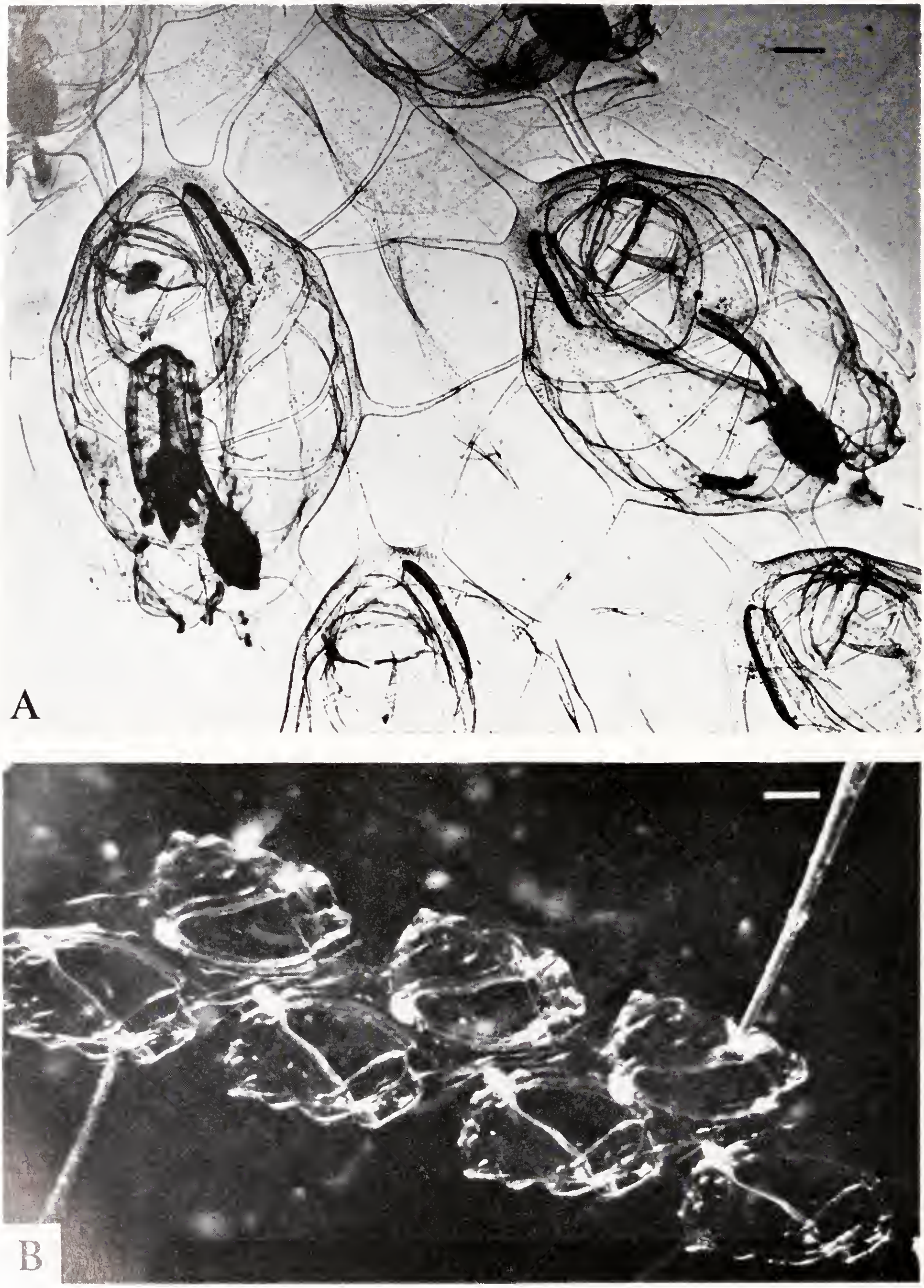

FIGURE 10. A. *Thalia democratica*, part of a chain of young blastozooids showing interconnections (photo by Claude Carré). Note oozoid embryo in the individual on the left

be strategically placed for sensing movement of adjacent zooids might function in locomotory coordination (Bone, 1959). Coordination could theoretically be achieved either by conduction of impulses between individuals or, mechanically, by tension or compression changes picked up by sense organs or excitable epithelia in each zooid in turn and relayed along the colony.

Electrical recordings have been made from intact salp chains restrained within a stockade of pins, or held by suction tubes (Fig. 10B). If a zooid in any part of the chain is stimulated so as to evoke OSPs, OSPs appear successively in other individuals along the chain (Fig. 11). In *Pegea* the transmitted response which followed a single shock was initially a burst of two or three OSPs rather than a single OSP, but after several stimuli, single OSPs were seen. The OSP burst may be similar in different zooids but, just as often, it varies either in the number of frequency relationships of the pulses. This argues against a simple through-conducting epithelial pathway, which should show a one for one relationship between stimuli and SPs and, judging by the behavior of SP systems within single zooids, should be less prone to fatigue and threshold changes than we find to be the case in chains.

By contrast, in stolons of *S. fusiformis,* OSPs are conducted in a simple one to one fashion with single electrical stimuli without obvious fatigue or threshold changes and with a short refractory period (18 msec).

There is some indication that "conduction velocity" (strictly speaking, the rate at which OSPs appear successively down the chain) changes as the chain matures. In a stolon, SPs were through-conducted at 5.25 cm/sec; in a very young chain grown from a stolon in the lab, the rate was 9.0 cm/sec; in slightly older chains retrieved from the plankton, 12.5 cm/sec. Mature chains of *Pegea* conducted OSPs at 5.25 cm/sec.

On the basis of present evidence, it appears probable that stolons and young chains possess epithelial through-conduction pathways which are later lost or modified. The spread of OSPs in older chains might represent sequential generation of these events in response to a signal mediated either by nerves or mechanically.

What, if any, causal relationship exists between the spread of skin pulses along the chain and the locomotory changes which also spread along the chain has not been determined, and until more is known about the precise role of the skin pulse systems at the level of the individual zooid, this question cannot be usefully pursued.

Discussion

This study has confirmed the main points in Fedele's account of locomotory organization in salps; namely, the timing of the firing sequence of the valve and body muscles as the basis for forward and reverse swimming, the neurogenic origin of the swimming rhythm, and the coordinated behavior of salp chains. We have shown further that the body wall muscles do not propagate but show graded respon-

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(scale is 1 mm). B. *Salpa fusiformis,* chain of young blastozooids with recording electrodes attached to the test. A salp so attached receives little stimulation from the recording electrodes and behaves much as in nature (scale is 1 cm).

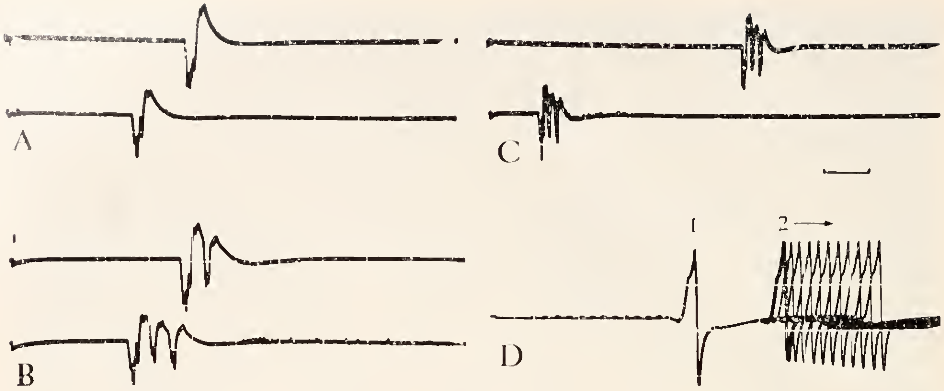

FIGURE 11. Recordings of outer skin pulses from salp chains (A-C, *Pegea confoederata*, D, stolon of *Salpa fusiformis*): A and B are recordings from two zooids separated by another zooid, simple (A) or complex (B) responses follow single shocks (scale is 200 msec); C. recording from two zooids with 20 zooids in between (scale is 500 msec); and D. two shocks were delivered every 10 secs to the stolon with the interval between the shocks being reduced by 5 msec at each sweep until the abosolute refractory period was reached sweeps are superimopsed, so that all first responss (1) coincide, while second responses (2 onwards) are spread out in time, (scale is 200 msec).

sivity, and we have recorded from a pacemaker cell and from presumed locomotory motoneurons in the brain. The brain resembles several other invertebrate ganglia in being able to generate a complex motor program without the need for peripheral input or feedback (Kater, Heyer and Kaneko, 1975).

It would be of considerable interest to clarify the mechanism whereby the switch from foward to reverse locomotion is achieved. The brain is capable of delaying the output to one end of the animal by a considerable interval (40 msec in the case of forward swimming). The photic responses of salps also deserve further study. The simple ON and OFF responses noted here could be mediated by much less complex photoreceptors than salps are known to possess and probably represent only a fragment of the total picture. Salp photoreceptors show hyperpolarizing receptor potentials as do those of vertebrates (Gorman, McReynolds and Barnes, 1971).

The major new feature of salp behavior to come to light in our work is the ocurrence of conducting epithelia. There are at least four independently conducting skin pulse systems. Their territories are distinct, as are their electrical signals. In other tunicates with conducting epithelia there is unequivocal evidence that skin pulses affect locomotory activity; each time a skin pulse is evoked, a change in locomotion follows. In the tadpole larva of one ascidian (*Dendrodoa*) SPs inhibit swimming (Mackie and Bone, 1976); in the larvacean *Oikopleura* they initiate or accelerate locomotion (Bone and Mackie, 1975). In the latter, uncoupling the skin from the central nervous system surgically or by drugs is the only way of preventing SPs from affecting locomotor activity.

In *Oikopleura* skin pulses enter the CNS through a pair of neurosensory receptors located in the skin. These receptors have bristle-like processes and can function as mechanoreceptors in the absence of skin pulses, affecting locomotion in the same way (Bone and Mackie, 1975, confirmed by recent, unpublished observations). The inhalent and exhalent valves of salps (*S. maxima*) are equipped with sensory receptors having long processes which reach to the surface of the test (Bone, 1959), and similar receptors occur elsewhere in the outer skin. On the larvacean model, these receptors would serve both as mechanoreceptors and as entry routes for skin pulses. Fedele (1933c) states that receptors are present in the inner lining of the pharynx, but we have not observed nerve endings in the gill or pharyngeal epithelia, where ISPs are propagated. Nerves may, however, be associated with the endostyle and peripharyngeal bands (Fedele, 1933a), and receptors in these regions might serve as ISP entry points.

In salps the pathway from skin to CNS seems to be more labile than in tadpoles and larvaceans, for skin pulses sometimes have no effect on locomotion. The salp system is certainly more complex than that of the smaller tunicates in that the locomotory response to skin stimulation can vary depending on where the stimulus is applied. ASPs evidently enter the nervous system anteriorly and cause locomotory arrest or reversal. OSPs are conducted over the entire outer epithelium and might therefore enter the nervous system at several different levels in succession, the order in which the respective neurosensory units were excited being a critical factor in determining the locomotory response. This however is entirely speculative. In the case of ISPs, there is no firm evidence regarding their function, and the situation is complicated here by uncertainty about the distribution of sensory endings.

Taking into account the work on larvaceans and ascidian tadpoles, it now appears that conducting epithelia are widely present in free-swimming tunicates, but to date there has been no satisfactory evidence for their existence in sessile ascidians. Of all the tunicates, the salps probably offer the best opportunities for analysis of the interactions beween conducting epithelia and nerves, and we hope to pursue our investigations in this direction.

It is a pleasure to thank the Director and staff of the Station Zoologique, Villefranche-sur-Mer, for their hospitality and assistance during the period of this research. We particularly thank J.-C. Braconnot for numerous suggestions and bibliographic help.

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SUMMARY

1. Various observations by M. Fedele on the mechanism of forward and reverse locomotion, on the neurogenic origin of the locomotor rhythm and on the coordinated behavior of salp chains are confirmed or extended. *Salpa fusiformis* was the species chiefly studied.

2. The striated muscle fibers of the body wall exhibit nonpropagative, graded responsivity. The fibers are multiply-innervated. Adjacent fibers are not electrically coupled.

3. Intracellular recordings are reported from a pacemaker and presumed motor neurons in the brain. The locomotor rhythm is exhibited by deafferented and isolated brains. In the intact animal, sensory input can modify the rhythm and alter the firing sequence of the muscles. The rhythm is accelerated by reduction, and inhibited by elevation of the ambient light intensity.

4. The outer skin is a conducting epithelium. The cells conduct action potentials at *ca.* 17 cm/sec and are connected by gap junctions. Three other independently conducting inner epithelial territories are described. Propagated impulses in the excitable epithelia are believed to enter the nervous system *via* neurosensory processes in the skin, extending the effective fields of these receptors.

5. Salp chains show coordinated responses but, except in their earliest developmental stages, impulses are probably not through-conducted along the chain, but are relayed from one zooid to the next by an unknown mechanism.

6. Comparisons are drawn between salps and other pelagic tunicates where conducting epithelia have previously been reported.

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A FINE-STRUCTURAL STUDY OF EMBRYONIC AND LARVAL DEVELOPMENT IN THE GYMNOBLASTIC HYDROID *PENNARIA TIARELLA*

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Investigations of development in hydrozoans have consisted for the most part of descriptive studies of normal development using light microscopy and general histological staining procedures. Specific histochemical methods have been employed, however, to monitor macromolecular changes during development of *Pennaria* (Cowden, 1965) and *Hydractinia* (Edwards, 1973). In addition, Edwards (1975) has used autoradiographic methods to investigate changes in nucleic acid and protein synthesis during development of *Hydractinia.*

Few studies on hydrozoans other than *Hydra* have extended observations to the fine-structural level. For certain Anthozoa, the planulae (Lyons, 1973; Vandermeulen, 1974; Chia and Crawford, 1977) and adults (Goreau and Philpott, 1956; Lyons, 1973; Chia and Crawford, 1977) have been so described. Fine-structural studies of polyps of Hydrozoa have been limited to analyses of cell types in *Campanularia* (Brock, 1970), the epidermis of *Cordylophora* (Jha and Mackie, 1967), and mechanoreceptors of *Coryne* (Tardent and Schmid, 1973). There has been no detailed account of fine-structural changes during development of a hydrozoan, although the surface coat of the planula of *Corydendrium* has been described (Glätzer, 1970); and limited use was made of the electron microscope in a comparative study of development in gymnoblastic hydroids (Van de Vyver, 1967).

The vast amount of new information gleaned from ultrastructural analyses of *Hydra* (for a review, see Burnett, 1973) suggested the potential value of examining at the fine-structural level embryonic and larval development in a hydrozoan more typical than *Hydra.* The marine hydroid *Pennaria tiarella* was chosen for such a study because of the morphological and histochemical information already available (Cowden, 1965; Summers and Haynes, 1969; Martin and Edwards, 1974).

MATERIALS AND METHODS

Colonies of *Pennaria tiarella* were collected from wharf pilings near the Bermuda Biological Station in July, 1973. Similar collections were made in August, 1975, at the Duke Marine Laboratory, Beaufort, North Carolina. Fronds from several mature male and female colonies were placed together in large finger bowls of Millipore-filtered sea water. The bowls were placed in the dark at 6:00 PM, and at 9:00 PM early cleavage stages were collected.

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Embryos were fixed at selected stages of development. The series collected in 1973 included the 2-hour cleavage, the 8-hour gastrula, and the 13-, and 16-hour planula stages. The series collected in 1975 was more complete, and included 2-, 4-, 6-, 8-, 10-, 12-, and 16-hour stages. Specimens were fixed for one hour at room temperature in 2% glutaraldehyde in 0.1 M sodium cacodylate-buffered sea water, pH 7.0, containing 1.5% sucrose and 0.01 M $CaCl_2$ (modified from Anderson and Personne, 1970). The embryos were rinsed in 0.1 M sodium cacodylate-buffered sea water, pH 7.0, containing 5% sucrose and 0.01 M $CaCl_2$ for three 10-minute changes at room temperature. They were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.0, for one hour, followed by a 30-minute rinse in 0.1 M buffer. The specimens were dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in an Epon-Araldite mixture. In the 1975 collection, a complete developmental series was also fixed for light microscopic study both in Clark's 3:1 ethanol-acetic acid fixative and in 10% neutral buffered formalin, dehydrated in an ethanol series and embedded in Paraplast Plus paraffin.

Specimens fixed for electron microscopy were sectioned with glass knives on a Porter Blum MT2-B ultramicrotome. Sections were placed on 200-mesh copper grids, stained in 3% uranyl acetate followed by 0.2% lead citrate (Veneable and Coggeshall, 1965), and examined with a Zeiss EM9S-2 electron microscope. Plastic sections 0.18 to 0.36 μ thick from a region of the block adjacent to that used for thin sections were mounted on glass slides and stained with 0.5% toluidine blue in 1% sodium borate for examination with the light microscope.

At each stage examined, paraffin sections 10 μ thick were utilized to obtain histochemical information and to make comparisons with results reported by other investigators. Paraffin sections were mounted with Mayer's albumin, hydrated through a series of alcohols in the usual manner, and stained.

General histological staining procedures included Mallory's Triple Stain, hematoxylin and eosin, and hematoxylin and Gray's Double Contrast Stain (Gray, 1958). Himes' Triple Stain (Thompson, 1966) was used to provide an overview of localization of deoxyribonucleic acid (DNA), protein, and mucopolysaccharide/glycogen.

Histological procedures for nucleic acids included toluidine blue, pH 4.0, with control slides incubated in hot trichloroacetic acid (TCA), deoxyribonuclease (DNAse), or ribonuclease (RNAse) according to the protocol of Thompson (1966); the nucleal Feulgen for deoxyribonucleic acid, with TCA or DNAse pretreatment of control slides (Thompson, 1966); and azure B for ribonucleic acids (RNA) with RNAse pretreated sections used as control slides (Swift, 1955). The periodic-acid-Schiff (PAS) procedure with α-amylase-treated control slides was employed for the localization of mucopolysaccharides (Thompson, 1966); and Best's carmine with α-amylase-pretreated control slides was used to identify sites of glycogen deposits (Thompson, 1966). The specific stain for the localization of protein was 1-fluoro-2, 4-dinitrobenzene coupled with β-naphthol with control slides pretreated with trypsin (Thompson, 1966).

In 1973, the living organisms were photographed with an Olympus photomicroscope just prior to their fixation. Paraffin sections were photographed with a Leitz Ortholux photomicroscope.

RESULTS

Two- to six-hour stages (cleavage)

From paraffin sections, it is obvious that at two hours post-fertilization, the blastomeres are of unequal size. In general, the outer blastomeres are smaller than the inner ones, and mitotic figures are more numerous peripherally. Cell nuclei stain positively with specific stains for DNA and RNA. Nuclear staining properties remain unchanged during development.

The cytoplasm contains both acidophilia and basophilia. Acidophilia is indicated by a positive reaction to fast green, aniline blue, and napthol yellow S. Positive staining with dinitrofluorobenzene-β-naphthol suggests that this acidophilic component includes protein. Basophilia is demonstrated by staining with hematoxylin. Staining of the cytoplasm with azure B, eliminated by RNAse pretreatment, indicates the presence of cytoplasmic RNA. The cytoplasm of the outer blastomeres stains more intensely with the specific stains for protein and RNA than does the cytoplasm of the central blastomeres.

In 2-hour embryos fixed in 10% neutral buffered formalin, the peripheral cytoplasm appears distinctly granular. The granules are acidophilic, as indicated by a strong affinity for such acid stains as fast green, aniline blue, Gray's Double Contrast Stain, and naphthol yellow S. That the acidophilia includes protein is indicated by staining of the granules with dinitrofluorobenzene-β-naphthol, employed as a specific stain for protein. The granules are also positive for periodic-acid-Schiff (PAS) and negative for Best's carmine, indicating that they contain mucopolysaccharide, but not glycogen. The absence of nucleic acid is indicated by the consistently negative response of the granules to staining with nuclear Feulgen, azure B, and toluidine blue.

The granules are not preserved in Clark's 3:1 fixative. The difference in preservation of the granules between those embryos fixed in formalin and those fixed in Clark's alcohol-acetic acid mixture is suggestive of a phospholipid component of the granules, since that is a class of biological macromolecules known to be both soluble in alcohol and rendered insoluble by formalin (Thompson, 1966; Galigher and Kozloff, 1971). A basophilic component of the granule, demonstrable by staining with hematoxylin, may represent this phospholipid, although acid mucopolysaccharides are also basophilic.

A thin glycocalyx on the free surface of each blastomere is positive for acid dyes and dinitrofluorobenzene-β-naphthol, an indication of a protein component. Positive staining with PAS reflects the presence of mucopolysaccharide and/or glycogen. Staining of the glycocalyx with Best's carmine, reduced in intensity by pretreatment with α-amylase, reveals that this surface coat does contain glycogen. Areas of PAS-positive material are also present between adjacent blastomeres.

In electron micrographs of 2-hour embryos, microvilli are arranged in a sparse brush border at the cell surface (Figs. 1 and 2a). Also detectable is a fibrillar-like substance, *ca.* 1.25 μ thick, coating the surface of the embryo. Ovoid, electron-dense, membrane-bound granules with diameters ranging from 0.4 to 0.8 μ are located directly beneath and in close association with the cell surface membrane (Fig. 2b). At this and later stages, the granules, which are easily identifiable in

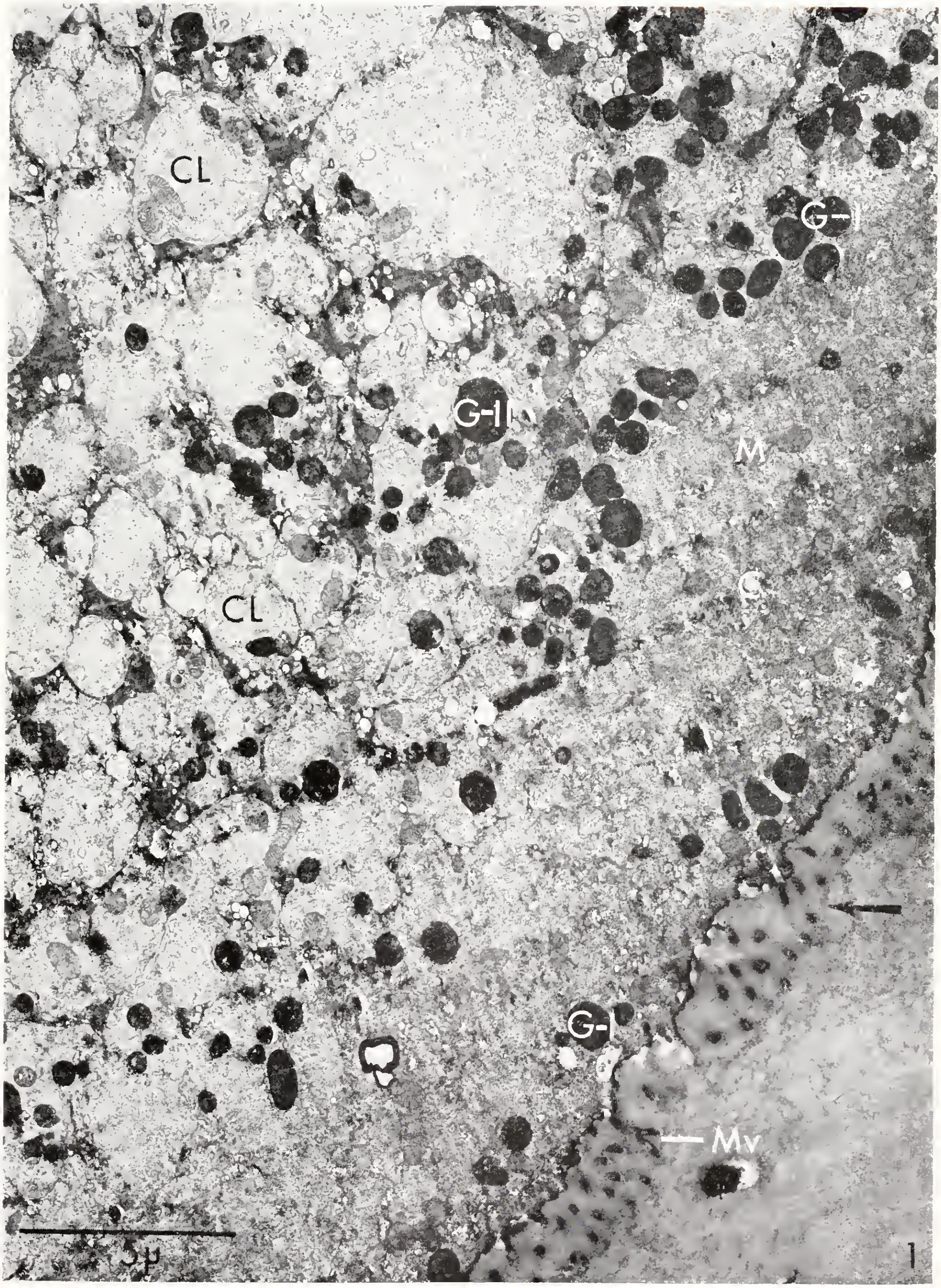


FIGURE 1. In the 2-hour stage a fibrillar-like substance (arrow) is associated with the microvilli. C represents cortex; CL, cytoplasmic lobule; G-I, Type I granule; G-II, Type II granule; M, mitochondrion; and Mv, microvillus.

thick plastic sections, are identical in size, shape, and location to the granules seen at the light microscopic level. These will be referred to as Type I granules.

A granule-free cortex, *ca.* 8 to 10 μ thick, separates the Type I granules at the surface from a band of similar granules occurring more interiorly (Fig. 1). Mitochondria with an unusual morphology are found scattered throughout the cortical region (Figs. 1 and 2c). These mitochondria have an extremely electron-dense matrix, and thick cristae measuring *ca.* 0.18 μ by 0.04 μ. Small, electron-dense bodies, *ca.* 0.02 μ in diameter, are found in the mitochondrial matrix (Fig. 2c).

Interior to the cortex of the blastomeres there occur anucleate, membrane-bound cytoplasmic lobules, measuring *ca.* 2 μ in diameter (Fig. 2d), which are identical in morphology and location to cytoplasmic lobules which can be seen in thick plastic sections. Lobulation of the cytoplasm is not discernible in paraffin sections, however. The cytoplasmic lobules are separated from one another by a homogeneous, electron-dense ground substance. The spheres contain the atypical mitochondria described above, Type I granules, an occasional Golgi body, free ribosomes, and short segments of granular endoplasmic reticulum. The organelles are dispersed in what appears to be a random arrangement within the cytoplasmic lobules. Large granules (Fig. 2e), *ca.* 0.8 μ in diameter, are found in some of the cytoplasmic lobules. These electron-dense Type II granules are characterized by the presence of numerous small, electron-lucent areas. No such granules can be detected in the cortical region.

Between two and four hours post-fertilization, morphological changes occur. In thick plastic sections, the surface of the 4-hour embryo appears ruffled. Microvilli have increased in length. The presence of surface vacuoles suggests that endocytosis or exocytosis is occurring. Type I granules have increased in number and have begun to accumulate at the surface in wedge-shaped pockets, *ca.* 1.5 to 2.0 μ in diameter. The circumferential granule-free cortical zone detected earlier is no longer present. Metachromatic granules (Type II), *ca.* 0.6 μ in diameter, appear in the central regions of the embryo. Some of the nuclei possess centrally located nucleoli. The nucleoli are more abundant in the inner blastomeres than in the outer ones.

At the fine-structural level, a few surface cilia can be seen. In the more peripheral cells the homogeneous, electron-dense ground substance found between cytoplasmic lobules at the 2-hour stage has disappeared, although the compartmentalizing membranes delimiting the cytoplasmic lobules still occur (Fig. 3a). Mitochondria increase in number, but retain the atypical morphology described above. In the cytoplasmic lobules (Fig. 3b), numerous segments of granular endoplasmic reticulum appear coalesced at the periphery of the spheres, located just beneath the limiting membrane. Free ribosomes, atypical mitochondria, and Golgi bodies occur in the lobules. Type I granules, first detected at the 2-hour stage, are present both in the cytoplasmic lobules and at the embryo surface. Numerous intercalations of the plasma membranes between adjacent blastomeres can be seen (Fig. 3a).

With the electron microscope it can be seen that by six hours of development the spherical intracellular organization of the cytoplasm into lobules is less obvious (Fig. 4a). Type I granules occur in increased numbers in aggregates near the surface. Type II granules are present in the deeper portions of the embryo. Microvilli are numerous and regularly arranged along the surface (Fig. 4b).

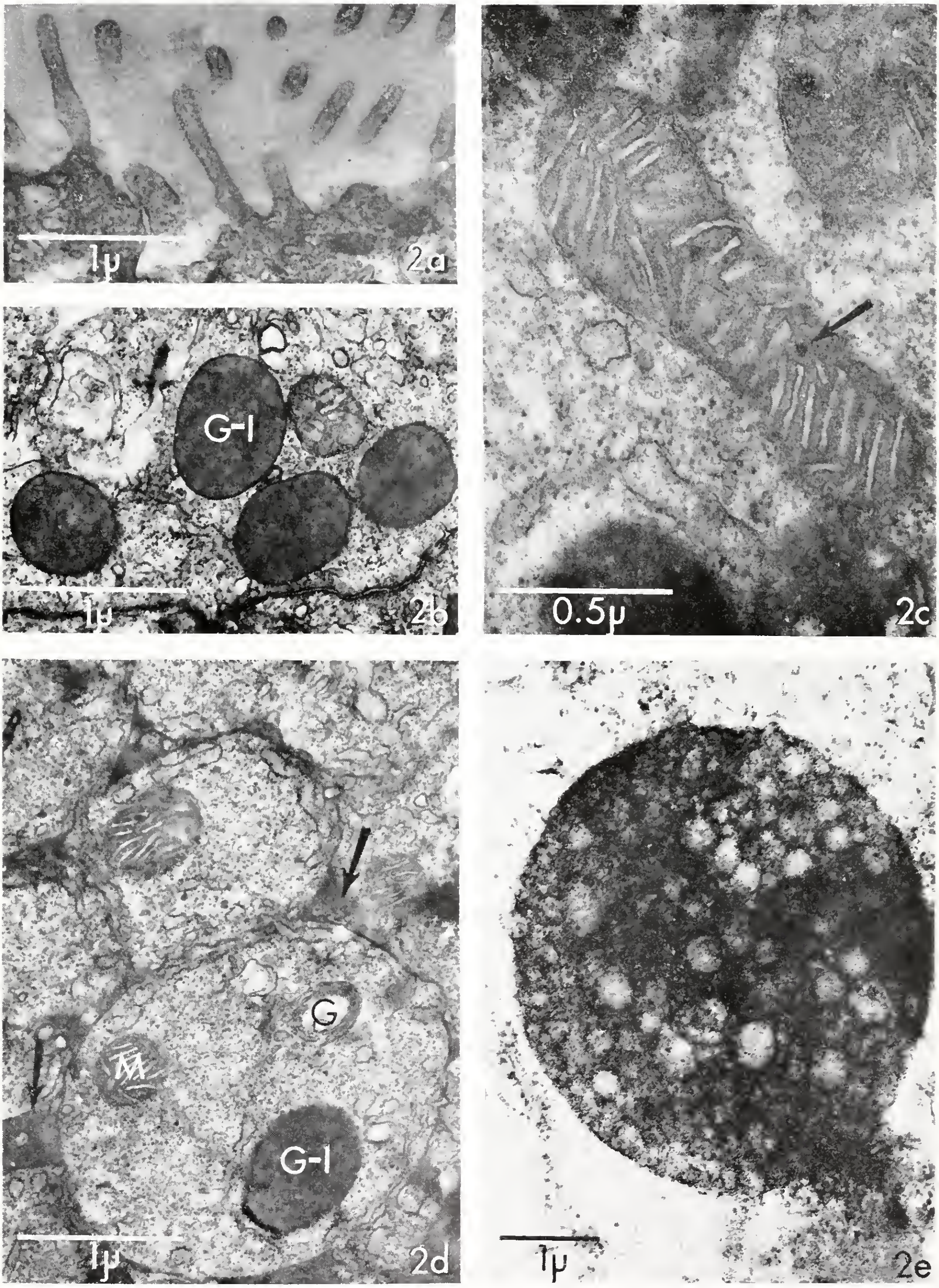


FIGURE 2. Embryos at the 2-hour cleavage stage possess: a. microvilli and a surface coat; b. Type I granules; c. mitochondria with electron-dense bodies; d. cytoplasmic lobules separated by an electron-dense ground substance (arrows); and e. Type II granules within cytoplasmic lobules. G represnts Golgi body; G-I, Type I granule; and M, mitochondrion.

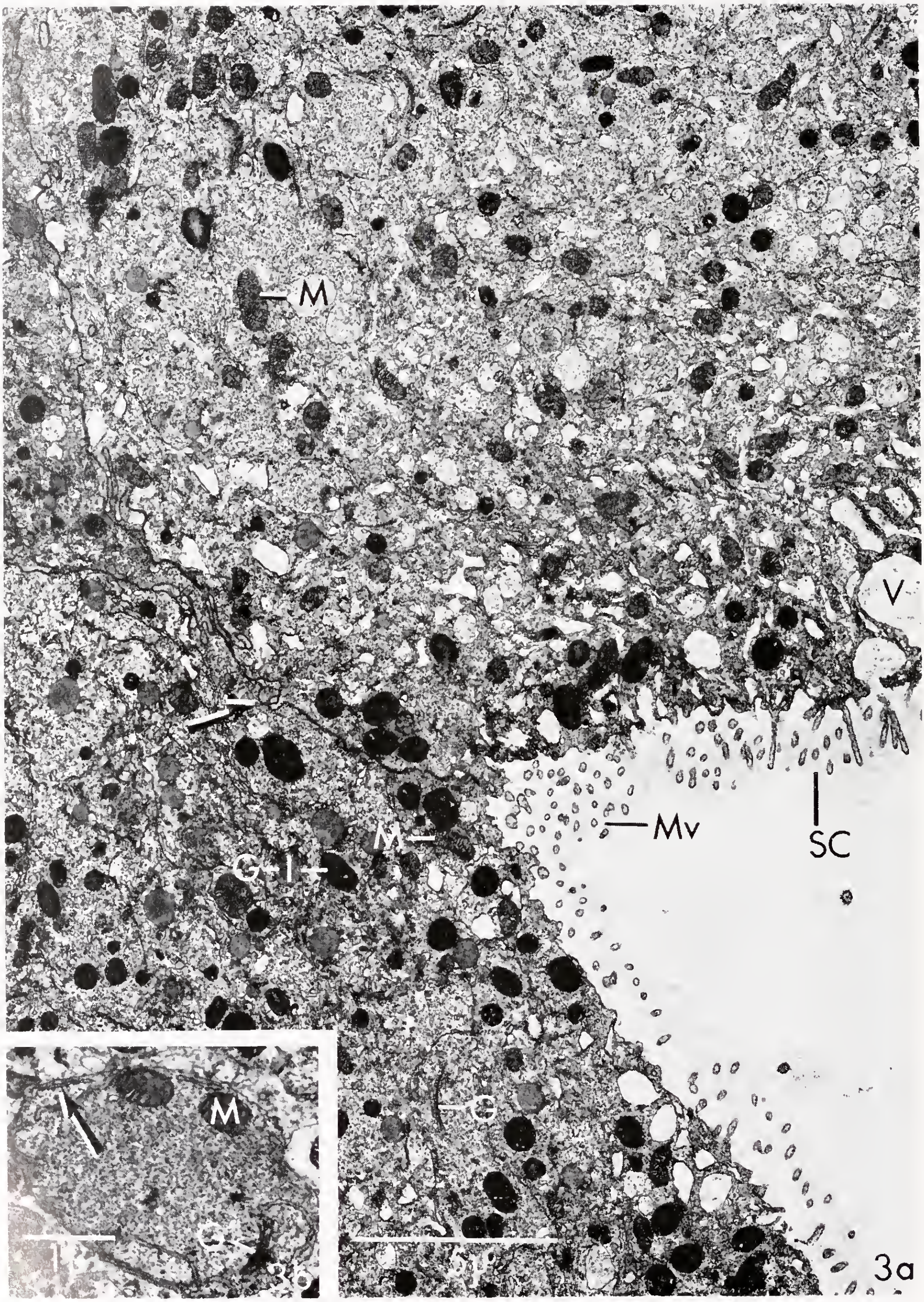


FIGURE 3. At the 4-hour cleavage stage: a. intercalations of plasmalemma between adjacent cells are abundant (arrow); and b. cytoplasmic lobules contain segments of granular endoplasmic reticulum coalesced beneath the limiting membrane of the lobule (arrow). G represents Golgi body; G-I, Type I granule; M, mitochondrion; Mv, microvillus; SC, surface coat; and V, vacuole.

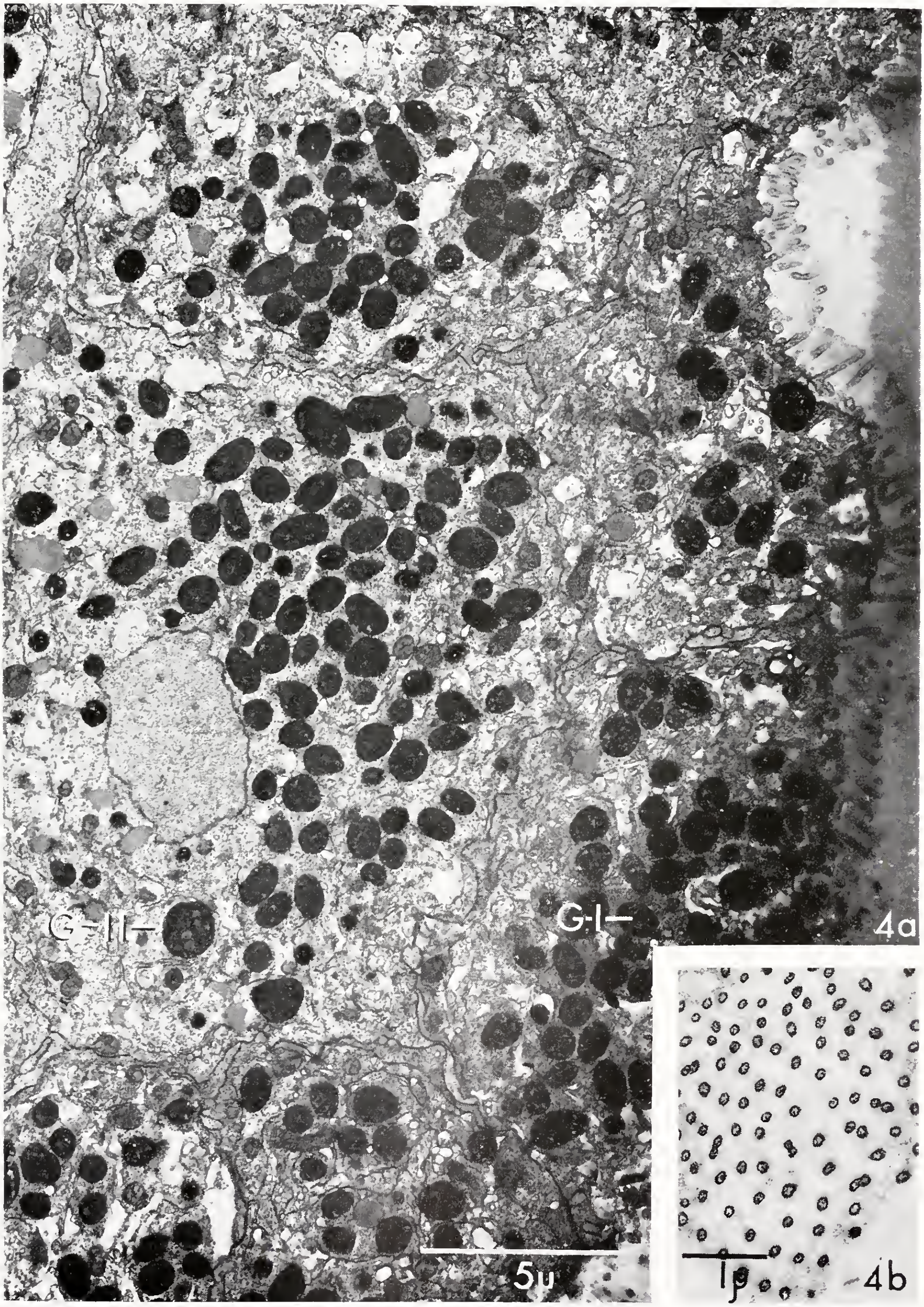
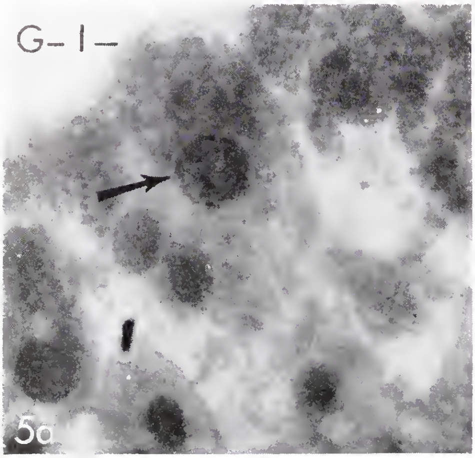
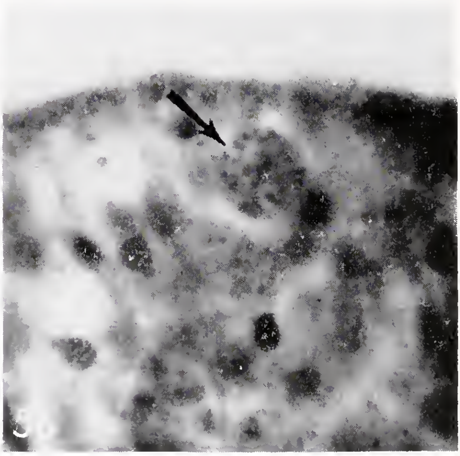
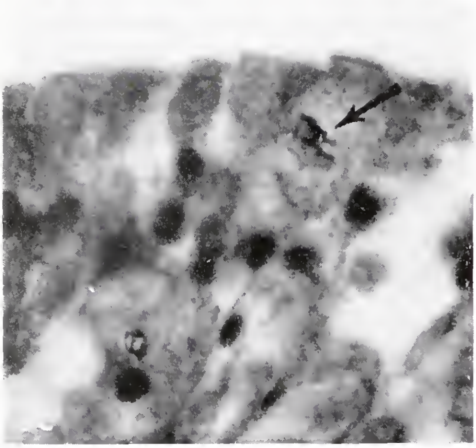
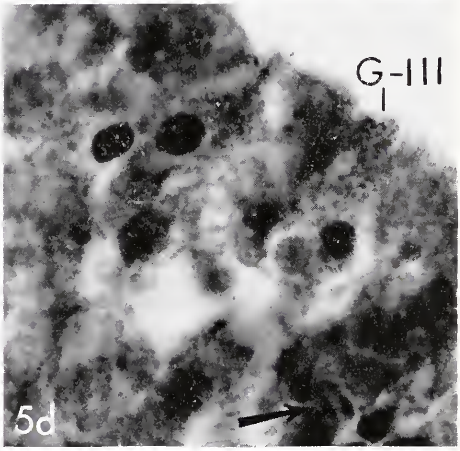


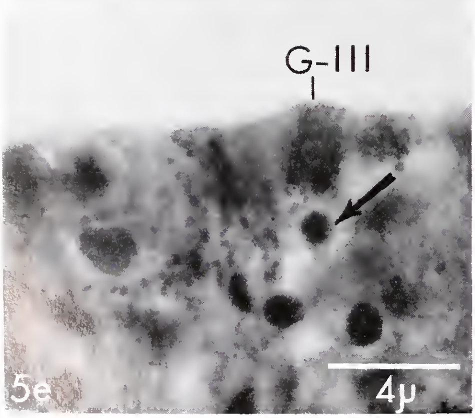
FIGURE 4. By six hours post-fertilization: a. the cytoplasmic lobules have disappeared; and b. microvilli are uniformly distributed over the surface of the cells. G-I represents Type I granule; and G-II, Type II granule.


G- I -
5a






G-III
5d


G-III
4μ
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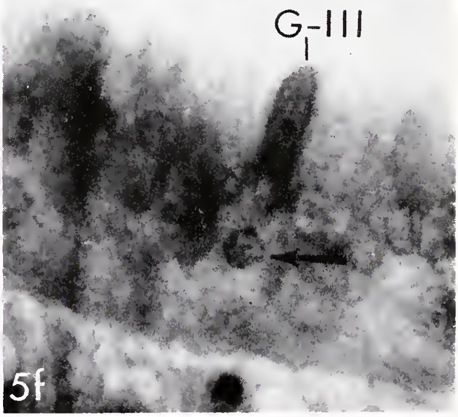
G-III
5f

Eight-hour stage (post-gastrulation)

Gastrulation is completed by the formation of an acellular mesoglea (Cowden, 1965). The mesoglea can be detected in paraffin section at the 8-hour stage, although it is too thin to be analyzed histochemically. At the fine-structural level, it appears relatively undeveloped, measuring *ca.* 0.2 μ in thickness. Few, if any, fibers can be detected in the mesoglea at this stage.

Concomitant with the appearance of the mesoglea, differentiative events can be detected in the ectoderm. By eight hours, numerous mitotic figures are seen in the extreme apical portion of the ectoderm (Fig. 5a–d). Following division, the cytoplasm of these cells contains mucous granules which stain strongly with Best's carmine. The Best's carmine-positive granules (Type III) occur in discrete pockets, with the nucleus of the cell located at the proximal tip of the pocket (Fig. 5e and f). With the electron microscope, the Type III granules, which have an average diameter of *ca.* 0.4 μ, appear as membrane-bound structures with an electron-lucent matrix (Fig. 7e).

The apical portions of the differentiating epithelio-muscle cells (Fig. 6), which constitute the majority of the cells, are little changed from the cells of the pregastrulation embryo. The microvilli, which are *ca.* 1.0 to 1.5 μ in length, have an unusual distribution at this stage, however, in that they occur in pairs, diverging at an acute angle from a common basal region (Fig. 7a and b). These pairs appear to be uniformly distributed at intervals of *ca.* 1 μ over the surface of the cells. Epithelio-muscle cells possess a single cilium (Fig. 7c) with the associated ciliary structures characteristic of cnidarians (for a review, see Chapman, 1974). Each cilium arises from a knob-like cytoplasmic projection which is *ca.* 0.2 μ in diameter. The apical portion of the basal body extends into this evagination. At the basal plate there is a constriction of the plasmalemma surrounding the cilium to a diameter of *ca.* 0.16 μ, above which, in the region of cilium proper, the diameter increases to *ca.* 0.23 μ. The constriction gives the cilium the stalked appearance typical of the cilia of many cnidarians (Jha and Mackie, 1967; Lyons, 1973; Vandermeulen, 1974; Chia and Crawford, 1977).

Large numbers of Type I granules occur peripherally, interspersed among numerous electron-lucent vacuoles (Fig. 6). Mitochondria are also a conspicuous feature of the apical cytoplasm. More internally, Golgi complexes and segments of granular endoplasmic reticulum predominate. Nucleoli are first detected in the peripheral cells at this stage.

In at least some of the cells, the cytoplasm is divided by two closely apposed membranes into parallel compartments (Fig. 7d). At intervals, the membranes separate to form electron-lucent vacuoles. Within the cytoplasmic compartments,

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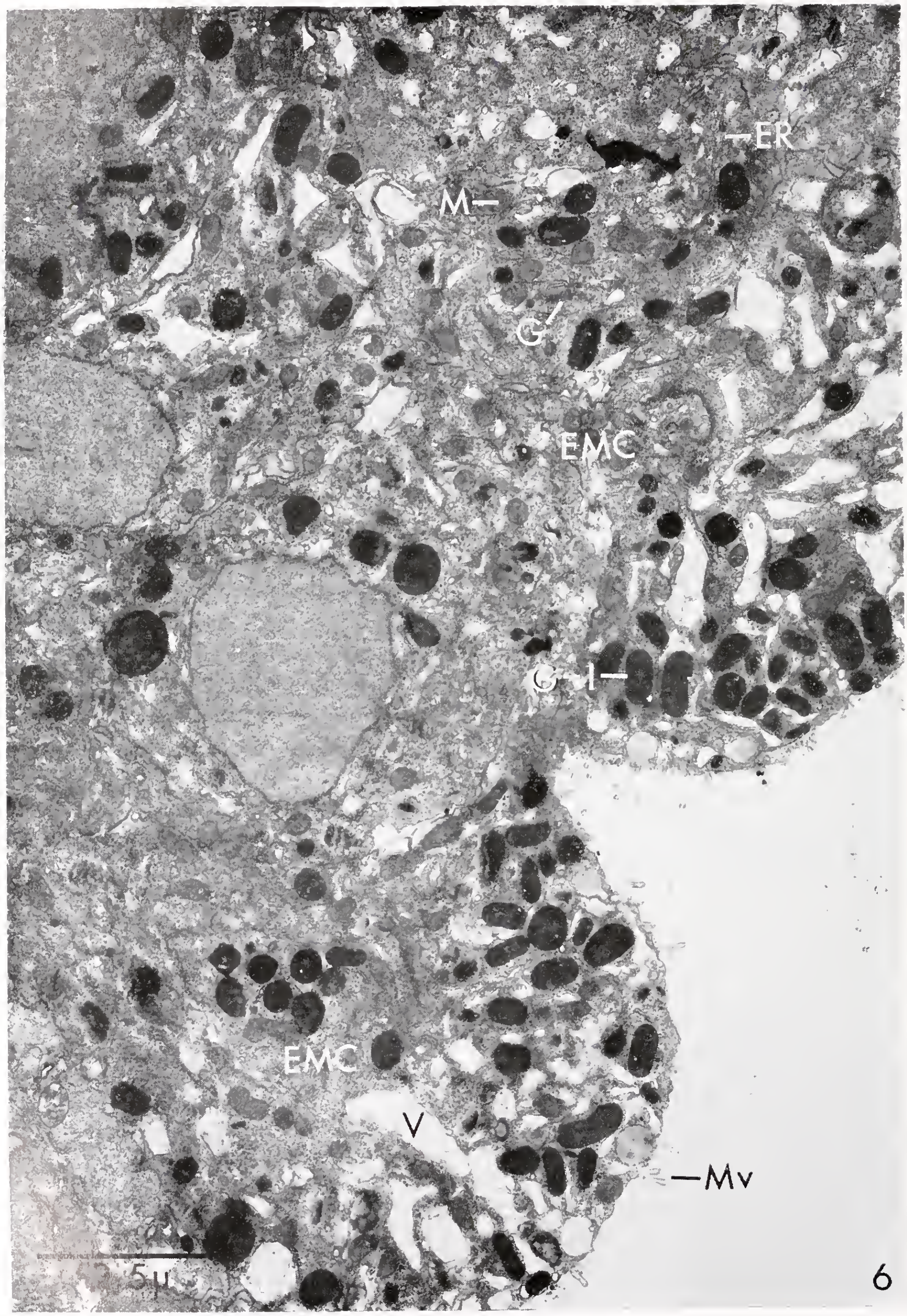
FIGURE 5. In light micrographs of the 8-hour stage, mitotic figures are detected in the apical portion of the ectoderm. The cytoplasm of these cells subsequently contains a third type of granule, Type III, which stains strongly with Best's carmine. a. The spherical cell contains fast green-positive Type I granules. The nucleus of the cell is indicated by the arrow. b. Spherical cells in prophase (arrow), c. metaphase (arrow), and d. telophase (arrow) can be detected. A third type of granule, Type III, appears in the ectoderm following division of the spherical cells. e. During differentiation of the mucous cell, the nucleus (arrow) lies at the proximal tip of the pocket of granules, and f. as development proceeds, the nucleus (arrow) becomes basally located. G-I represents Type I granule; and G-III, Type III granule.


ER
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6

there frequently occurs a single cisterna of granular endoplasmic reticulum. Golgi complexes are often associated with and oriented parallel to the longitudinal compartments. Type I granules and mitochondria are scattered throughout the cytoplasm in these areas.

Type II granules are seen in the lower basal portions of the epithelio-muscle cells. The basal portions of the epithelio-muscle cells have begun to differentiate into foot processes which abut on the mesoglea, but these processes are incompletely differentiated in that myonemes cannot be detected.

Sixteen-hour stage (planula)

By 16-hours of development, the mesoglea has increased in size and complexity. It is *ca.* 0.3 μ thick and is composed of tiny, mesh-like fibers dispersed in an amorphous ground substance.

Three cell types can be detected at the 16-hour stage (Fig. 8). Especially numerous near the anterior end of the planula are mucous cells containing Type III granules, which first appeared at the 8-hour stage, and which stain positively with both Best's carmine and PAS and negatively with fast green (Fig. 8a and b). The pockets of granules extend more deeply into the ectoderm than was the case in the earlier stages; and the nuclei, located at the proximal ends of the pockets, lie in the basal half of the ectoderm (Fig. 8a). One or two large, clear, refractile vacuoles (Fig. 8a) often occur in association with these nuclei.

By 16 hours, epithelio-muscle cells are well-developed. The cells are columnar, extending from the free surface of the embryo to the mesoglea. The nuclei are elongated and are located medially with respect to the long axis of the cell. The nuclei are often surrounded by small unstained vacuoles. Type I granules, unchanged histochemically (Fig. 8a and b) and morphologically (Fig. 9) from those described for earlier developmental stages, heavily infiltrate the apical portion of the cells. The cytoplasm contains numerous small, electron-lucent vacuoles near the apex; short, sparse strands of granular endoplasmic reticulum; mitochondria; and free ribosomes. In the basal regions of these cells, large Type II granules can be seen (Fig. 10a).

Basal foot-processes of epithelio-muscle cells abut on the surface of the mesoglea at this stage, but myonemes have not completely formed. Atypical mitochondria are scattered throughout these foot processes.

The third cell type, the nongranular cell, first detected histochemically at the 10-hour stage, shows a strong affinity for dinitrofluorobenzene-β-naphthol, aniline blue, and Gray's Double Contrast Stain (Fig. 8c). These cells are evenly distributed around the periphery of the embryo. The nuclei are elongated and are slightly more basally located than are the nuclei of the epithelio-muscle cells.

The nongranular cell as it appears by electron microscopy is shown in Figures 9 and 10. The cells are characterized by having an unusual type of cytoplasmic compartmentalization in the area surrounding the distal half of the nucleus and

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FIGURE 6. The apical portion of the 8-hour postgastrulation embryo is characterized by the presence of electron-lucent vacuoles and Type I granules near the surface. EMC represents epithelio-muscle cell; ER, endoplasmic reticulum; G, Golgi body; G-I, Type I granule; M, mitochondrion; Mv, microvillus; and V, vacuole.

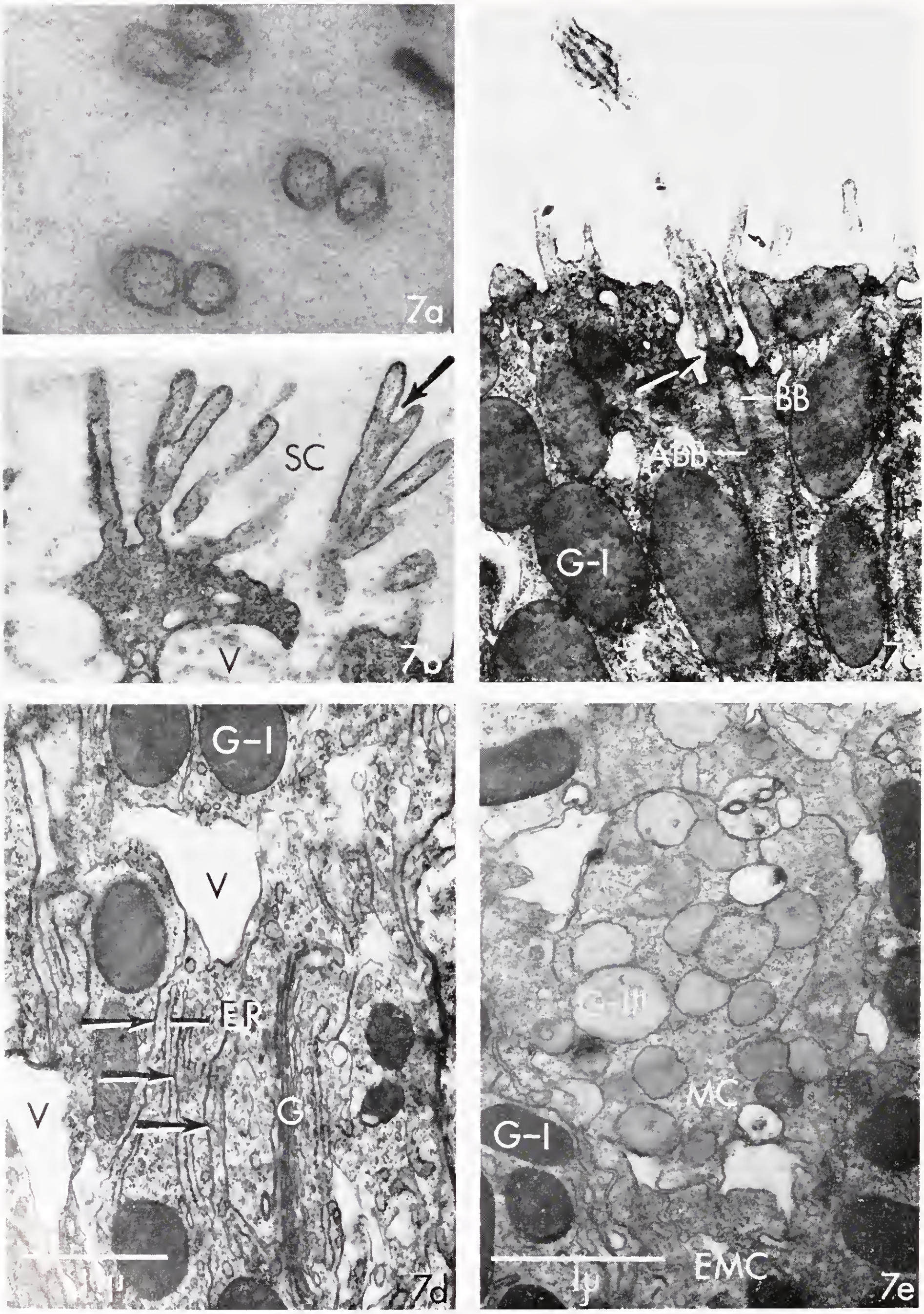
7a
SC
V
7b
BB
ABB
G-I
7c
G-I
V
ER
V
G
1μ
7d
G-I
G-III
MC
G-I
1μ
EMC
7e

FIGURE 7. Several features characterize the 8-hour embryo. Microvilli occur in pairs as can be seen a. in transverse section, and b. in longitudinal section. The two microvilli

extending *ca.* 16 μ toward the apex of the cell. In this region, cell membrane oriented parallel to the long axis of the cell divides the cytoplasm into long, slender compartments (Fig. 10a). Each compartment typically contains either a single segment of granular endoplasmic reticulum or a series of segments aligned end-to-end. The endoplasmic reticulum is swollen at intervals to give a beaded appearance (Fig. 10b). Electron-dense accumulations *ca.* 0.1 μ in diameter occur within the cisternae of the endoplasmic reticulum (Fig. 10c). The membrane responsible for compartmentalization is modified from the typical unit membrane found elsewhere in the cell. That the modification may involve fusion of adjacent unit membranes is indicated by areas in which the unusual membrane is continuous with two discrete unit membranes (Fig. 10d). The cytoplasmic organization resembles that described in certain cells at the 8-hour stage (compare Figs. 7d and 10b).

These cells are also characterized by the presence along their entire lengths of electron-dense particles measuring only *ca.* 0.08 μ in diameter (Fig. 10e). The particles are not delimited by membrane; rather, they are surrounded by a halo of extremly electron-lucent material. The particles may occur singly, but as many as eight have been seen within a single area of electron-lucent cytoplasm. The non-granular cells also contain Type II granules near the mesoglea.

Fewer intercalations between adjacent ectodermal cells can be observed at 16 hours. Gap junctions *ca.* 0.1 μ in diameter can be detected, however.

DISCUSSION

The cytoplasmic organization of the pregastrulation embryo of a hydrozoan has never been reported. Electron micrographs of very early cleavage stages of *Pennaria* have revealed the presence of microvilli uniformly distributed over the surface of the embryo. The fibrillar material shown in the present study to be associated with the microvilli closely resembles the mucous coat of *Hydra* described at the fine-structural level by Davis (1973). The fibrillar material presumably represents then the morphological equivalent of the PAS-positive surface coat first demonstrated in embryos of *Pennaria* by Cowden (1965).

The time of the first appearance of cilia has not been reported previously for *Pennaria*. Their detection in the present study at the 4-hour stage suggests that ciliogenesis in *Pennaria* is precocious relative to other hydrozoans, since, among most hydrozoans studied to date, cilia never appear prior to gastrulation (Van de Vyver, 1967). It should be noted, however, that in the present study the first cilia, which were present in small numbers, could not be detected with light microscopy at the 4-hour stage. This suggests that what appears to be precocious development of cilia in *Pennaria* may reflect instead the sensitivity of the method used to examine the embryo.

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are seen to share a common basal region (arrow). c. Cilia, present on the epithelio-muscle cells, have a constriction of the plasmalemma at the basal plate (arrow). d. The cytoplasm of certain cells is divided by fused membranes into parallel, longitudinal compartments (arrows) containing single cisternae of granular endoplasmic reticulum. e. Mucous granules occur in pockets in the ectoderm. ABB represents accessory basal body; BB, basal body; ER, endoplasmic reticulum; EMC, epithelio-muscle cell; G, Golgi body; G-I, Type I granule; G-III, Type III granule; MC, mucous cell; SC, surface coat; and V, vacuole.

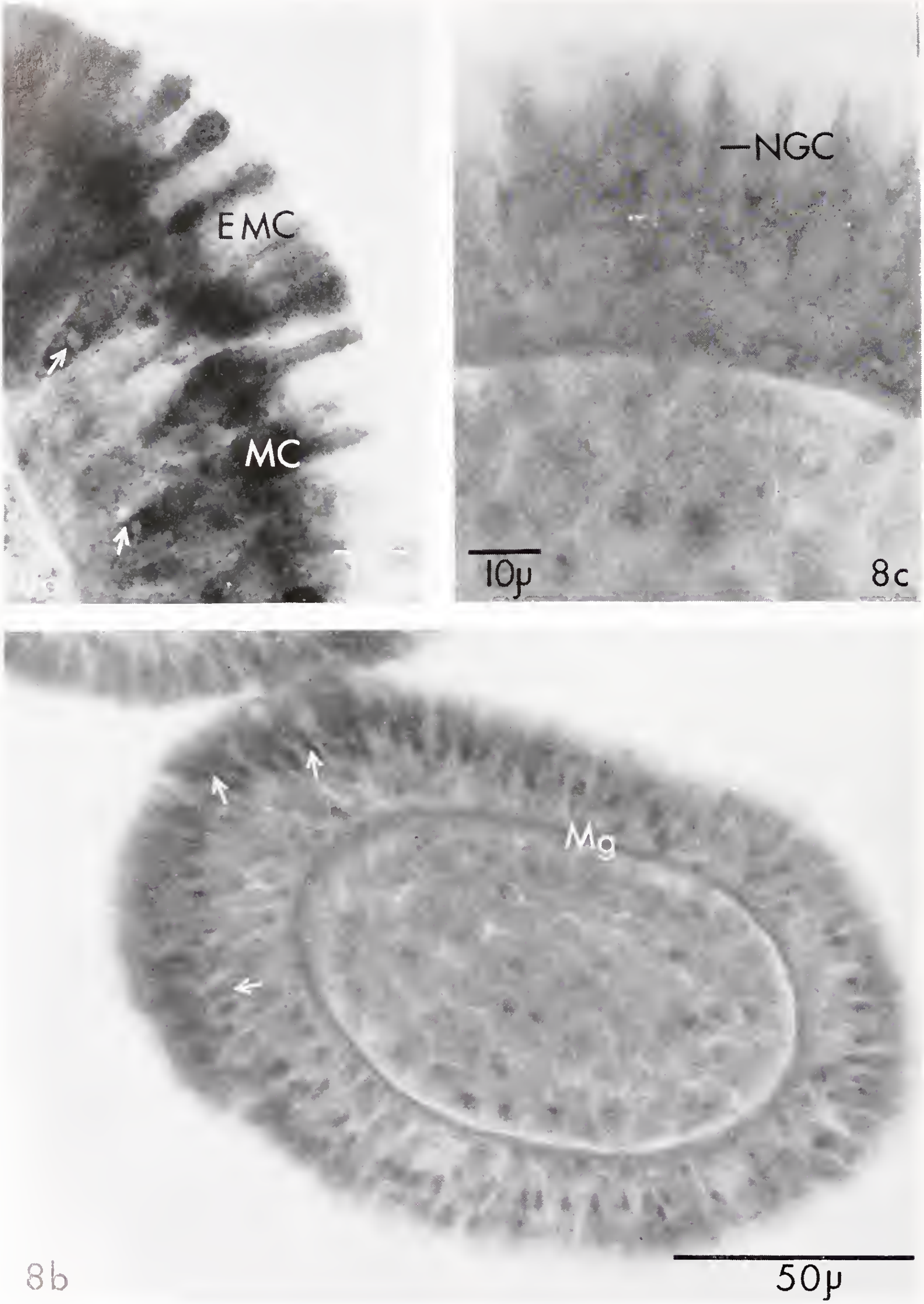
EMC
MC
NGC
10μ
8c
Mg
8b
50μ

FIGURE 8. Histochemical analyses of the 16-hour planula reveal the presence of three cell types: epithelio-muscle cells, mucous cells, and nongranular cells. a. Stained with Best's

In addition to providing new information concerning surface structures of the pregastrulation blastomeres, electron microscopic examination has revealed an unusual organization of their internal cytoplasm. The cells at the very early stages were seen to contain spherical, membrane-delimited lobules separated from one another by an amorphous ground substance which disappeared by the 4-hour stage. Since the ground substance was not discernible in paraffin sections, its composition is not known.

The origin of the cytoplasmic organization of certain cells following gastrulation into parallel compartments delimited by fused unit membrane and containing a single cisterna of endoplasmic reticulum may be reflected in the organization of the pregastrulation blastomeres. The cytoplasmic lobules seen in the 2-hour stage persist following the disappearance of the ground substance. Their internal structure becomes highly organized with a single cisterna of granular endoplasmic reticulum occurring along a portion of the periphery of the lobule. Fusion of membranes of adjacent lobules, together with elongation of the lobules as the blastomeres elongate, could result in the unusual cytoplasmic compartmentalization described for later developmental stages. It is also possible that fusion of membranes of adjacent lobules might effect elongation. In this regard, it should be mentioned that microtubules, which are often found in association with elongating cells, were not detected in elongating cells of *Pennaria,* even though the usual precautions were taken to insure their preservation.

The membrane-bound, electron-dense Type I granules, which were present in large numbers in the earliest developmental stages examined, and which persisted during larval development, have not been previously reported for *Pennaria.* That these granules were not detected in the study by Cowden (1965) can be attributed to the fact that they were not preserved in Clark's 3:1 fixative which was employed in that study. The Type I granule demonstrated in formalin-fixed material in the present study has some of the staining properties that were apparently interpreted as general cytoplasmic staining by Cowden. In that study it was reported that cytoplasmic levels of PAS-positive and azure B-positive materials were relatively high during early cleavage. The same results were obtained in the present study in embryos fixed in Clark's solution, and no Type I granules could be discerned. In formalin-fixed embryos, however, it appears that the PAS-positive material is associated with discrete granules. The azure B-positive material, on the other hand, is associated with the general cytoplasm and not with the Type I granule.

Large, heterogeneous vitelline granules have been demonstrated in the eggs and developing embryos of *Hydractinia, Clava,* and *Cordylophora* (Van de Vyver, 1964, 1967), but from the descriptions and micrographs presented these bear no resemblance to the small, homogeneous Type I granules which occur in *Pennaria.* They

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carmine, the ectoderm shows two cell types. Epithelio-muscle cells are Best's carmine-negative, whereas mucous cells are Best's carmine-positive. The pockets of mucous granules extend more deeply into the ectoderm than was the case in earlier stages. The nuclei, located basally, often have clear, refractile vacuoles associated with them (arrows). b. The mucous cells (arrows) are also PAS-positive, as is the mesoglea. c. A third cell type evenly distributed around the periphery of the embryo, and first detected at the 10-hour stage, stains strongly with dinitrofluorobenzene-β-napthol. EMC represents epithelio-muscle cell; MC, mucous cell; Mg, mesoglea; and NGC, nongranular cell.

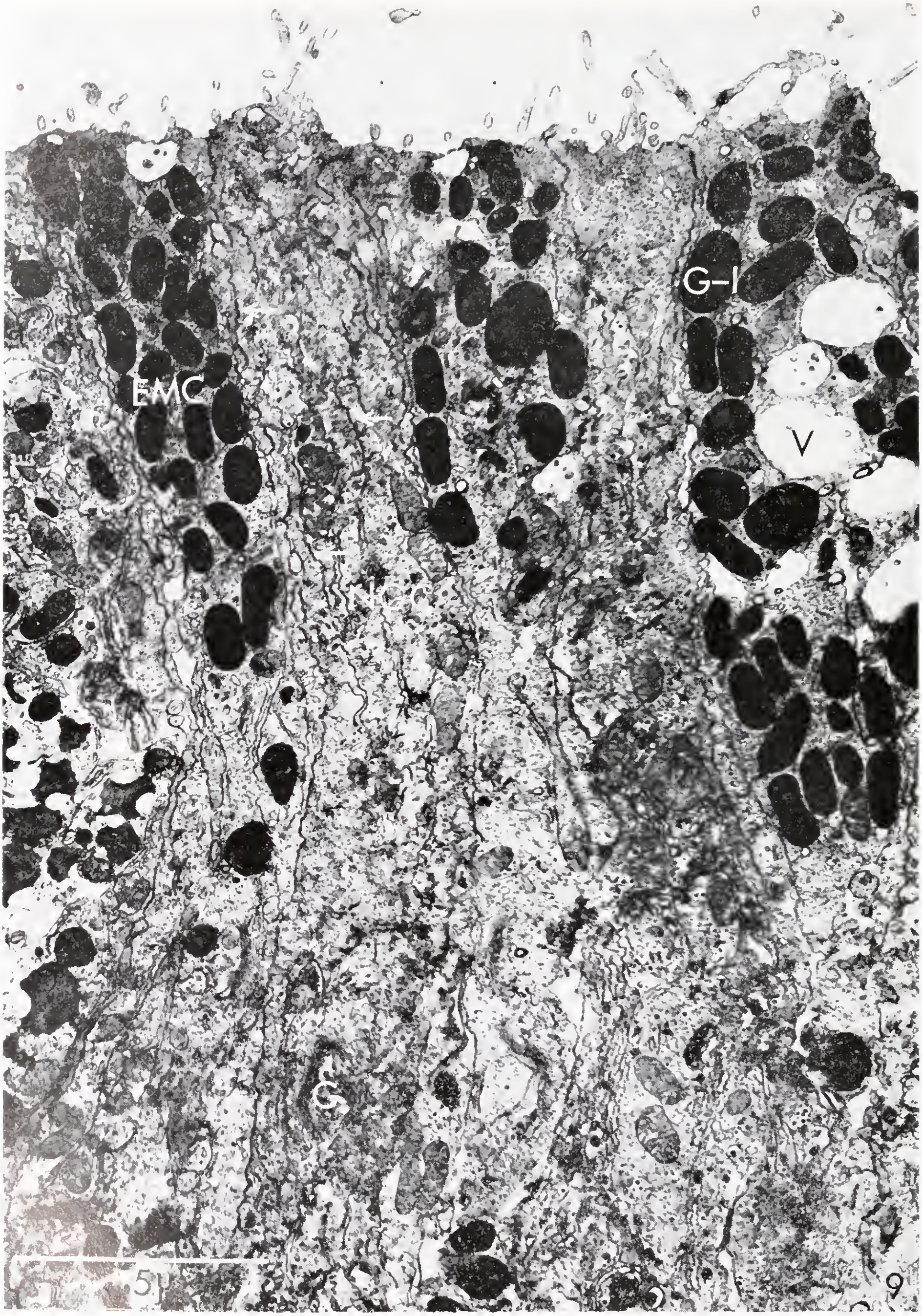

FIGURE 9. Sections of the ectoderm reveal differences between the apex of the nongranular cell and the epithelio-muscle cell. The nongranular cells contain long segments of

do resemble in size and electron density the degenerating mitochondria shown in a micrograph of *Hydractinia* by Van de Vyver (1967). In this regard, according to Van de Vyver (1967), Ephrussi has described for embryos of *Clava* a process of formation of lipid reserves for the embryo through modification of mitochondria. Although there is a similarity in size between mitochondria and Type I granules in *Pennaria,* it is unlikely that the granules represent degenerating or modified mitochondria, since no granules intermediate between mitochondria and Type I granules have been observed at any stage of development in *Pennaria.* Moreover, a function of the Type I granule as a food reserve appears unlikely, since, in contrast to the fate of the yolk granules described by Van de Vyver (1967), there is no significant decrease in their number in the epithelio-muscle cells during the course of larval development.

Haynes (1973) described the epithelio-muscle cell of *Hydra* as a developmentally polarized cell, with the apical region modified for secretory activity, and has suggested that large, electron-dense secretory droplets located apically are involved in the production of the surface coat. In the larvae of *Pennaria,* the epithelio-muscle cells are polarized and a surface coat does exist, even at the earliest stages examined. Type I granules occur apically, are also present at the earliest stages examined, and exhibit some of the staining properties of the surface coat. Despite these facts, it should be pointed out that there was never any morphological evidence that membranes of the Type I granule fuse with the plasmalemma, and therefore no evidence for a secretory role for the Type I granule. Moreover, although a well-developed surface coat is associated with mature polyps of *Pennaria,* Type I granules have never been observed in the polyp (unpublished observations). The presence of these granules throughout embryonic and larval development and their absence in the adult suggest that they play a role in metamorphosis. The possibility of such a function is currently being investigated.

Ectodermal mucous cells have been described for the planulae of many hydrozoans, including *Pennaria* (Cowden, 1965; Summers and Haynes, 1969). The mucous cells of *Pennaria* appear to be identical histochemically and morphologically, as well as in their ontogeny, to the mucous cells which function during the attachment of the planulae in all of the hydrozoans examined by Van de Vyver (1967). The origin of these cells from the products of mitotic activity at the apex of the ectoderm has not been reported previously.

It has been assumed that prior to migration of nematoblasts and interstitial cells from the endoderm only two cell types, the epithelio-muscle cell and the mucous cell, occupy the larval ectoderm of hydrozoans, although Van de Vyver (1967) has detected a second mucous cell type in the planula of *Coryne.* Examination of thick and thin plastic sections of the 16-hour planula of *Pennaria* has revealed the presence of an additional cell type, the nongranular cell. The cell is extremely difficult to detect in paraffin sections, even when it is known to exist from ultrastructural studies, and is clearly identifiable only in sections stained with dinitrofluorobenzene-β-naphthol. That the cell is not simply a degranulated

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granular endoplasmic reticulum (arrows). Epithelio-muscle cells, containing Type I granules, possess numerous vacuoles near the surface. EMC represents epthelio-muscle cell; G Golgi body; G-I, Type I granule; NGC, nongranular cell; and V, vacuole.

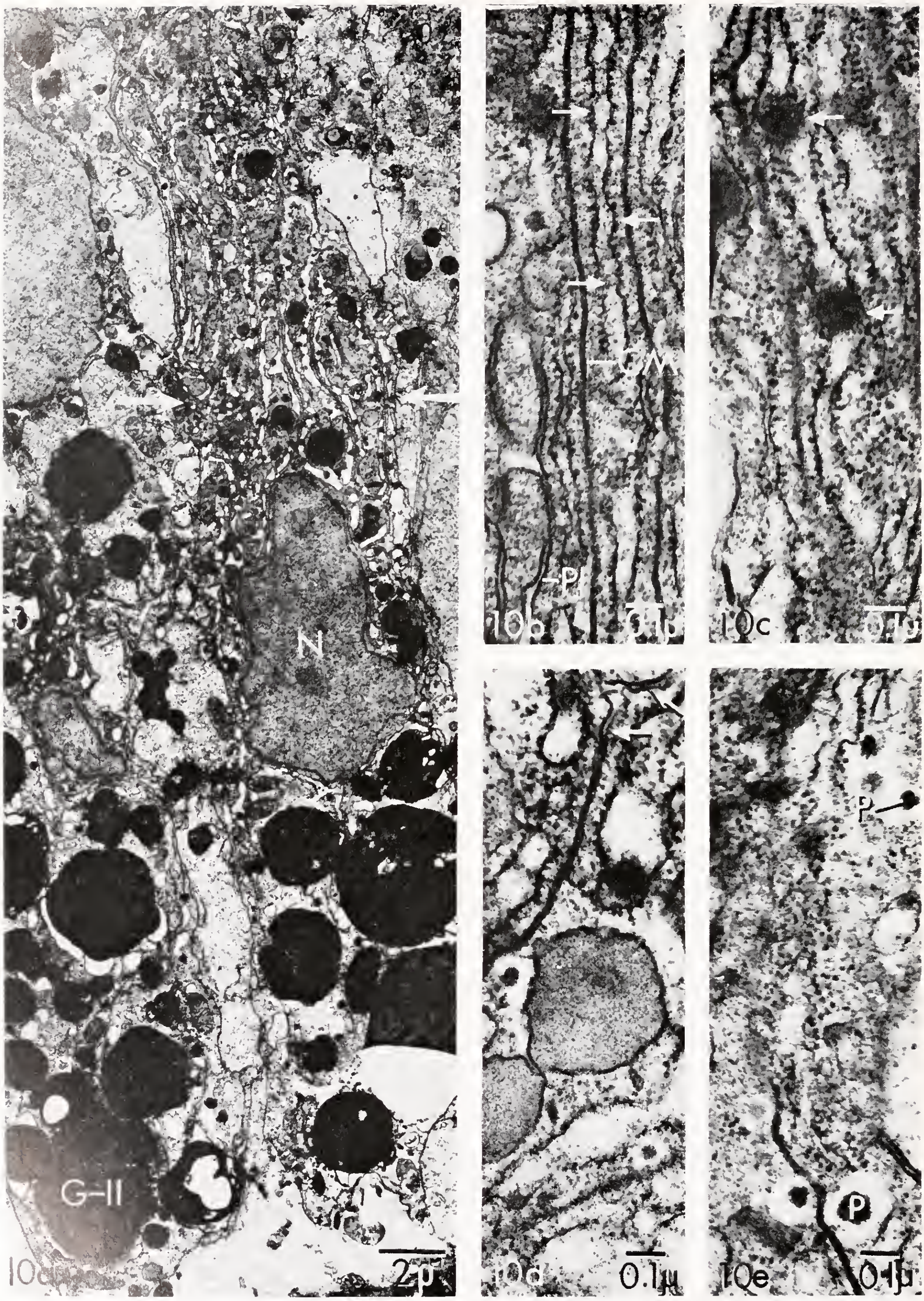
N
G-II
10a
2μ
CM
P
10b
0.1μ
10c
0.1μ
10d
0.1μ
P
P
10e
0.1μ

FIGURE 10. Sections of the medial and basal portions of the ectoderm demonstrate the features of the nongranular cell. a. An unusual cytoplasmic compartmentalization (arrows)

epithelio-muscle cell is indicated not only by its unique staining properties, but also by its distinctive fine-structural morphology. The nucleus is more electron-dense than that of the epithelio-muscle cell and often contains two nucleoli. The granular endoplasmic reticulum, located in longitudinal compartments distal to the nucleus, is swollen at intervals to surround spherical, electron-dense accumulations which also occur in membrane-bound vesicles in the adjacent cytoplasm. In addition, the cytoplasm contains numerous small, electron-dense particles surrounded by an electron-lucent zone. The vesicles and particles are never observed in the epithelio-muscle cells of *Pennaria.* Comparisons of the cytology of the nongranular cell of the planula of *Pennaria* with cell types described in *Hydra* (Davis, 1973; Westfall, Yamataka and Enos, 1971; Westfall, 1973) suggest that the cell most closely resembles a sensory cell, a possibility that is currently being investigated.

SUMMARY

1. The pregastrulation blastomeres contain electron-dense granules which become localized after gastrulation in the apices of the developing epithelio-muscle cells and persist throughout larval development. The cytoplasm of the blastomeres is organized into anucleate, membrane-delimited lobules. The lobules, which persist until six hours of development, come to contain a single, peripherally located cisterna of granular endoplasmic reticulum. Microvilli are present at the earliest stages examined and persist throughout development. Cilia are first detected at four hours.

2. Gastrulation, marked by the appearance of the mesoglea, occurs between six and eight hours of development. Basal foot processes of epithelio-muscle cells are detected by eight hours, but myonemes cannot be detected until later in development.

3. Immediately following gastrulation, mucous cells begin their differentiation from dividing cells located near the apex of the ectoderm. During their differentiation, the cells elongate toward the mesoglea.

4. By 16 hours post-fertilization, a third cell type can be detected in the ectoderm. The cell, which contains no granules, has an unusual cytoplasmic organization in which fused membranes divide the cytoplasm into parallel compartments containing a single cisterna of granular endoplasmic reticulum.

5. The findings of the present study are correlated with those of previous studies of development in *Pennaria* and other hydroids. The possible functional roles of the Type I granules, the cytoplasmic lobules, and the nongranular cell are discussed.

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is seen in the area surrounding the distal half of the nucleus. b. The granular endoplasmic reticulum within the compartments is swollen at intervals to give a beaded appearance (arrows). Note the thickness of the compartmentalizing membrane relative to that of the endoplasmic reticulum and the plasmalemma. c. Electron-dense accumulations (arrows) are present within the cisternae of the endoplasmic reticulum. d. An atypical membrane is responsible for compartmentalization. That the modification of the membrane may involve fusion of two adjacent unit membranes is indicated by the occurrence of regions in which the specialized membrane is continuous with two unit membranes (arrows). e. Electron-dense particles, not bounded by membrane, occur along the entire length of the nongranular cell. CM represents compartmentalizing membrane; G-II, Type II granule; N, nucleus; P, particle; and Pl, plasmalemma.

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AGGLUTININS AND LYSINS IN THE MOLLUSCAN FAMILY PLANORBIDAE: A SURVEY OF HEMOLYMPH, EGG-MASSES, AND ALBUMEN-GLAND EXTRACTS

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Substances which agglutinate or lyse human and other mammalian erythrocytes occur naturally in the hemolymph and in extracts from various tissues of molluscs. The function of these substances has not been determined; however, Prokop and his associates (1968a, b) assume that they play a role in the defense mechanisms of gastropods and coined the term "protectin". Pemberton (1974) noted that agglutinins have been detected in nearly 80 species of gastropods, including some species of Planorbidae (Boyd, Brown, and Boyd, 1966; Brown, Almodovar, Bhatia, and Boyd, 1968; Gilbertson and Etges, 1967; Lee-Potter, 1969; Pemberton, 1971; Rudolph, 1973).

Gilbertson and Etges (1967) observed differences in the distribution of hemolymph hemagglutinins in species and strains of *Biomphalaria* and suggested that these substances might be of value in differentiating populations of planorbids. In view of the limited number of strains employed in their study, Gilbertson and Etges' suggestion requires further evaluation. In fact, Pemberton (1974, p. 104) reviewed the literature on variation in terrestrial gastropods and concluded that, "There is, however, no current evidence that agglutinin content and specificity are of taxonomic value at species level."

In the present study, an effort was made to assess both interspecific and interpopulation differences in members of the Family Planorbidae with respect to the presence and specificity of agglutinins and lysins. Extracts of albumen glands and egg-masses, as well as hemolymph, were tested from 31 strains representing 8 species and 4 genera of Planorbidae.

MATERIALS AND METHODS

Snails were reared and maintained at 26 ± 1° C in 1.5, 5, or 10 gallon aquaria and fed romaine lettuce. The water was aerated and filtered, and the snails were exposed to fluorescent light for 12 hr daily. Species and strains of snails, their geographic origins and date of collection are listed in Table I.

Preparation of samples

Snail hemolymph samples were obtained by methods previously described (Michelson, 1966), and each sample represented a pool of hemolymph from 4–8 snails (12–15 mm in diameter).

To prepare egg extracts, egg-masses were removed from sheets of plastic film which had been placed previously in stock aquaria. No effort was made to separate the masses with respect to age or stage of development. The masses were rinsed in distilled water, blotted on paper toweling, and weighed on a micro-balance. They were then placed in a tissue-grinder and 5.5 μl of 0.85% saline added for each milligram of egg-mass. The contents were homogenized by hand for approximately 5 min, then centrifuged at 300 *g* for 5 min, and the supernate used as the test-sample.

To prepare albumen-gland extracts, albumen glands were removed from 5–10 mature snails (12–15 mm in diameter), weighed, and homogenized in a tissue-grinder containing 25 μl of 0.85% saline per milligram of tissue. The homogenate was centrifuged at 300 × *g* for 5 min, and the supernate used as the test-sample.

Hemagglutination tests

Serial doubling dilutions of test-material were prepared in U-bottom wells of Microtiter® plates (Cooke Engineering Co., Alexandria, Virginia). Dilutions were made in 0.85% phosphate-buffered saline (pH 7.2) at a final volume of 0.25 ml per well. An equal volume of a 2% human red-blood cell suspension was then added, the plates were covered, mixed on a mechanical shaker, and allowed to sit undisturbed for one hr at 27° C or overnight at 4° C. Control wells contained only the diluent and red-blood cells. Unless otherwise noted, a minimum of three different samples of each extract or hemolymph was tested for every snail strain. Test samples were used within one hour of preparation.

Studies, comparing agglutinins and lysins contained in the hemolymph and extracts from various species and strains of Planorbidae, utilized A_1, B, and O erythrocytes from the same series of three donors. To test for the possible variability in the receptor-cells, forty different samples of erythrocytes were obtained from either members of the Department of Tropical Public Health or from the Transfusion Center of the Children's Medical Center, Boston, Massachusetts. This collection of erythrocytes contained samples from twelve A_1, four A_2, three A_1B, six B, and fifteen O-type donors. The cells were subsequently used in tests employing albumen-gland extracts from seven strains of *B. glabrata*.

Since hemolymph and tissue extracts were pooled from series of snails of the various strains in the survey study, it was necessary to determine the degree of variability that might occur among individuals of a particular strain. Consequently, hemolymph from twenty-nine snails of the S-3 strain of *B. glabrata* and from ten snails of the CB strain of *H. caribaeum* was individually tested against A_1-type cells obtained from a single donor. In addition, extracts of individual albumen glands from ten snails of the S-3 strain were tested against A_1-type cells from a single donor.

Characteristics of hemolymph and tissue extracts

A preliminary and limited effort was made to characterize the physicochemical properties of the agglutinins contained in hemolymph and tissue extracts. Thus, albumen-gland extracts and egg extracts from two strains of *B. glabrata* (B-1 and S-3) were subjected to heat inactivation at 56° C and 80° C for one hr; dialysis against TrisHCL buffer (pH 7.8) for 24 hr; freezing at −20° C for up

TABLE I

Geographic origins of snail species and strains.

Species and strains	Origin and date of colonization
Biomphalaria glabrata	
PR-1	Puerto Rico, March 1954
PR-2	Rio de la Plata, Puerto Rico, December 1957
PR-2′	Strain derived from single egg of a PR-2 snail, April 1959
PR/B*	Puerto Rican/Brazilian hybrid, March 1966
B-1	Catinga do Moura, Bahia, Brazil, June 1964
B-2	Salvador, Bahia, Brazil, June 1964
S-3	Lake Amaralina, Salvador, Brazil, July 1964
BH*	Belo Horizonte, Minas Gerias, Brazil, July 1964
G-1	Fazenda Graviel, Castro Alves, Bahia, Brazil, June 1974
G-2	Fazenda Graviel, Castro Alves, Bahia, Brazil, June 1974
G-3	Fazenda Graviel, Castro Alves, Bahia, Brazil, June 1974
M-1	Fazenda Morro do Afonso, Castro Alves, Bahia, Brazil, June 1974
RS-1	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
RS-2	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
RS-3	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
RS-8	Fazenda Riacho Seco, Castro Alves, Bahia, Brazil, June 1974
St.L.	Cul de Sac Valley, Castries, St. Lucia, March 1969
Biomphalaria straminea	Recife, Pernambuco, Brazil, May 1964
Helisoma caribaeum	
257	Capella Viaja, Puerto Rico, July 1959
CH	San Juan, Puerto Rico, December 1970
DB	Dos Bocas, Puerto Rico, July 1959
CB	Castle Burke, St. Croix, Virgin Islands, August 1959
B	Bogota, Colombia, December 1970
Helisoma anceps	
H′ (pigmented and albino)	(?) Strain originated from tropical fish aquarium, September 1954
GB	Grand Bahama Island, June 1970
Bulinus truncatus	
E-1	Egypt, June 1957
E-2	Liberian Institute (? Egypt), December 1963
Bulinus globosus	
N-1	Nigeria, August 1972
SA-1*	South Africa, November 1965
Polypylis hemisphaerula	Liu Ying, Tainen, Taiwan, June 1963

* Albino strain.

to one yr; and repeated freezing and thawing for ten consecutive times. Extracts so treated were titered against A_1 erythrocytes and compared to freshly prepared extracts. In addition, attempts were made to block the agglutinating activity of albumen-gland extracts from B-1 and PR-2′ strains of *B. glabrata* by incorporating 0.1 M concentrations of various sugars in 0.85% saline in hemagglutination tests employing A_1 cells. Sugars used in this series of tests were as follows: N-Acetyl-D-Galactosamine, α-D(+)Fucose, L(−)Fucose, D(+)Galactose, β-D(−)Fructose, α-D(+)Glucose, α-Lactose, D(+)Maltose, D(+)Mannose, L(−)Sorbose, and Sucrose. These sugars were used also in attempts to block the agglutination of A_1, B, and O erythrocytes by hemolymph from *H. caribaeum* (CB).

RESULTS

Survey of strains and species

Egg extracts. Agglutinins for one type or another of human erythrocytes were detected in egg extracts from all seventeen strains of *B. glabrata* (Table II). The specificity of the reactions allowed a grouping of the strains into two categories: those whose agglutinins reacted only against A-type cells (B-2, S-3, BH, G-3, St.L), and those whose agglutinins reacted against both A- and B-type cells. The type of reaction observed did not appear to be correlated with albinism. Undiluted samples from strains B-1 and RS-2 agglutinated O-type cells; however, for practical purposes, reactions initiated by undiluted samples may be ignored. *B. straminea* agglutinated only A-type cells.

Except for extracts from the albino varient of *H. anceps* (H′) and from the E-2 strain of *B. truncatus*, in which undiluted samples agglutinated A-type cells and A- and B-type cells, respectively, agglutinins were not detected in the samples from the other strains and species.

Albumen-gland extracts. Albumen-gland extracts were prepared from all geographic strains of *B. glabrata* except the G-1 strain. Again, the reactions permitted the grouping of snails into two categories (Table III); those which react solely with A-type cells and those which react with both A- and B-type cells. The PR-1 strain showed slight reaction (1:4) in some samples, but not all, with O-type cells. Concentrated extracts of two strains gave evidence of lysis, but extracts of others did not.

TABLE II

Agglutinins detected in extracts of egg-masses from Biomphalaria *strains and species.*

Species and strains	RBC specificity and maximum titer		
	A_1	B	O
Biomphalaria glabrata			
PR-1	16	32	0
PR-2	64	64	0
PR-2′	32	64	0
PR/B	32	8	0
B-1	256	64	1
B-2	32	0	0
S-3	64	0	0
BH	64	0	0
G-1	8	16	0
G-2	8	16	0
G-3	16	0	0
M-1	16	16	0
RS-1	16	16	0
RS-2	8	8	1
RS-3	16	32	0
RS-8	16	32	0
St.L.	8	0	0
Biomphalaria straminea	64	0	0

TABLE III

Agglutinins detected in extracts of albumen-glands from strains of Biomphalaria glabrata.

Strains	RBC specificity and maximum titer		
	A_1	B	O
PR-1	32	32	4
PR-2	16	16	nd*
PR-2′	16	16	0
PR/B	32	1	0
B-1	16	4	0
B-2	32	0	0
S-3	128	0	0
BH	32	0	0
G-1	nd	nd	nd
G-2	16	16	0
G-3	16	4	0
M-1	8	32	0
RS-1	8*	8	*
RS-2	8	16	0
RS-3	8	8	0
RS-8	4	8**	nd
St.L.	64	1	0

* nd = not done.
** Signifies lysis in undiluted sample.

Extracts were prepared from three strains of *H. caribaeum* (257, CB, B), but no agglutinins were detected. The small size (4–5 mm) of *Polypylis* precluded the use of albumen-gland extracts and a whole snail extract was used. All tests were negative.

Hemolymph. Agglutinins were detected in the hemolymph from only six strains of *B. glabrata* (S-3, BH, G-2, G-3, M-1, B-2), and all had titers less than 1 : 8 except for the S-3 strain (1 : 32). A-type cells only were agglutinated by hemolymph from strains S-3, BH, and B-2; whereas, hemloymph from strains G-2, G-3, M-1 was reactive only against O-type cells. The S-3 strain gave higher titers at 4° C than at 27° C; titers for other strains were higher at 27° C. It is of interest that no Puerto Rican strain exhibited agglutinins, an observation first noted by Gilbertson and Etges (1967). Lysins were found in the hemolymph of all strains at either 4° or 27° C. Titers were generally low and rarely exceeded 1 : 2 in tests conducted at 27° C and only up to 1 : 16 in samples tested at 4° C. The lysins were nonspecific and reacted with all cell types.

Agglutinins in high titer were detected in six strains of *Helisoma* (Table IV). Strain 257 of *H. caribaeum* had titers up to 1 : 2048, the highest observed in any freshwater snail and possibly the highest hemolymph titer for any snail studied thus far. The agglutinins, however, showed no specificity and reacted with all types of cells. Lysins were observed in all strains except CH, B, and GB, and their titers were slightly higher than those of *Biomphalaria.*

In the genus *Bulinus,* only a South African strain of *B. globosus* gave evidence of having agglutinins. The titers were low and never exceeded 1 : 4.

TABLE IV

Agglutinins in the hemolymph of Helisoma *species and strains (27° C).*

Species and strains	RBC specificity and maximum titer		
	A_1	B	O
Helisoma caribaeum			
257	2048	256	256
DB	64	32	8
CH	0	0	0
CB	256	256	32
B	512	256	32
Helisoma anceps			
H′ (pigmented)	16	16	16
H′ (albino)	32	16	8
GB	0	0	0

Variability of receptor cells

Some variability was detected in the response (titer) of albumen-gland extracts when tested against cells obtained from a variety of donors (Table V) and those from the pools used in the survey of species and strains; however, strains in both studies reacted in a similar manner, and the results of both studies were comparable. Those strains that were reactive to A cells alone remained so, as did those which previously showed reactivity to both A- and B-type cells. No reactions were noted with O-type cells at titers greater than 1 : 1. Although the number of A_1B and A_2 donors was limited, it was observed from the material available that extracts from all strains aggluinated A_1B cells at titers from 1 : 8 to 1 : 64. A_2-type cells were either not agglutinated or reacted only at very low titers, usually less than 1 : 4.

Variability of extracts and hemolymph from individual snails

Although differences in titer were observed among hemolymph samples from individuals of CB strain of *H. caribaeum* and in extracts of the albumen-glands from individual S-3 snails, all snails in both groups responded uniformly in demonstrating the presence of agglutinins. In the *Helisoma* samples, all tests showed titers of 1 : 256, which was as far as the material was diluted. Titers from the albumen-glands, on the other hand, ranged from 1 : 8 to 1 : 128 with a geometric mean titer of 1 : 45.3. Hemolymph samples from S-3 snails were highly variable, some samples showing the presence of agglutinins (up to 1 : 64), whereas others were negative.

Preliminary characterization of agglutinins

Neither heat inactivation at 56° C, dialysis, nor repeated freezing and thawing appeared to effect the agglutinating activity of egg or albumen-gland extracts. Extracts frozen for up to three months at −20° C showed no loss of reactivity and those frozen for one year only showed a loss of one titer.

A remarkable difference was noted in the response of *Helisoma* hemolymph and

Biomphalaria albumen-gland extract to the presence of various sugars in the test system. N-Acetyl-D-Galactosamine effectively blocked agglutination of A_1-, B-, and O-type cells by *Helisoma* hemolymph, whereas the other sugars had no effect. On the other hand, N-Acetyl-D-Galactosamine had no effect on albumen-gland extracts from strains of *B. glabrata.* Albumen-gland extracts were markedly inhibited by α-D(+)Fucose, α-L(−)Fucose, β-D(−)Fructose, D(+)Mannose, L(−)Sorbose, D(+)Maltose, α-D(+)Glucose, and Sucrose. Partial inhibition was exerted by D(+)Galactose and α-Lactose.

Discussion

Hemagglutinins and hemolysins for human erythrocytes are not uniformly present in members of the family Planorbidae, and differences were observed between genera and species as well as between populations of a single species. The ability to detect these substances depends, in part, on the selection of the sample to be tested, and variations were noted between hemolymph, albumen-gland extracts, and extracts of egg-masses. Thus, lysins were found primarily in hemolymph samples, rarely in albumen-gland extracts, and never in egg-mass extracts. When hemolymph served as the test sample, agglutinins were detected in only 6 of the 17 *B. glabrata* populations; however, all populations exhibited agglutinins in egg and albumen-gland extracts. If one assumes that the albumen-gland is the source of agglutinins and that these substances are sequestered into developing eggs, as well as disseminated into the surrounding hemolymph, then this apparently erratic distribution can be explained. Failure to identify agglutinins in the hemolymph of some populations could be due to "spill-over" concentrations too low to detect

Table V

Agglutinins detected in extracts of albumen-glands from strains of Biomphalaria glabrata: *summary of trials employing RBC's from various donors.*

Strains	RBC specificity and mean titer*		
	A_1	B	O
BH	27.8 (10)** 8–64	0 (6) 0–1	0 (15)
PR/B	26.5 (11) 4–128	5.0 (6) 2–8	0 (15)
S-3	46.7 (11) 16–256	0 (6) 0–2	0 (13)
PR-2′	20.7 (8) 8–128	12.7 (6) 4–32	0 (15)
G-2	8.7 (8) 4–32	12.7 (6) 4–32	0 (15) 0–2
B-2	47.8 (11) 8–256	0 (4) 0–1	0 (12) 0–1
B-1	19.0 (8) 8–64	5.7 (6) 2–32	0 (15) 0–1

* Geometric mean and range.
** Numbers in brackets equals number of donors tested.

by the techniques employed. On the other hand, agglutinins were not found in either egg or albumen-gland extracts of any of the species of *Helisoma*. However, agglutinins were detected in the hemolymph of some strains and species and at very high titers. This suggests that the origin of the agglutinins in *Helisoma* may be different from those of *Biomphalaria*. The lack of specificity of the hemolymph agglutinins also distinguishes them from those of *Biomphalaria*. Moreover, when the results of the inhibition studies are considered, differences between the agglutinins of the two genera become patently apparent; N-Acetyl-D-Galactosamine effectively blocks *Helisoma* agglutinins, but has no effect on those from *Biomphalaria*. Likewise, those sugars which inhibited *Biomphalaria* agglutinins failed to be effective against agglutinins from *Helisoma*.

Although the limited number of species and strains of *Bulinus* and *Polypylis* tested prevents a definitive evaluation of these genera, it appears likely that both groups lack agglutinins specific for human erythrocytes. Brown *et al.* (1968) noted some reactivity with extracts from *Bulinus truncatus* when enzyme modified cells were employed, and the hemolymph of some *Bulinus* species appear capable of agglutinating nonhuman mammalian cells (Rudolph, 1973).

Gilbertson and Etges (1967) reported that the agglutinins found in the hemolymph of *Biomphalaria* lack specificity and reacted identically against A, B, and O-type cells. Our data appears to contradict theirs in that reactions occurred with either A or O-type cells, but not with both and not at all with B-type cells to the exclusion of A. These differences are not easily explained, since Gilbertson and Etges' snail strains from Bahia, Brazil were derived from our S-3 and B-2 colonies. Techniques differed in the type of hemoagglutination test employed, but this alone does not necessarily account for the differences observed. It may be suggested that the observed differences are due to variability of the erythrocytes obtained from different donors, but this does not agree with our experience. It should be noted, that the present study indicated that *Biomphalaria* hemolymph is a poor source of agglutinins, extremely variable, and not suitable alone for comparing species and strains.

The present study lends support to the thesis that lectins (agglutinins and lysins) may be a valuable tool in characterizing snail populations and aiding in the taxonomic discrimination of freshwater snail species. When egg or albumen-gland extracts were employed as a source of agglutinins, populations of *B. glabrata* could be segregated into two major groups—those which reacted with A-type cells and those that reacted with both A- and B-type cells. Further characterization could be made employing data on hemolymph lysins and agglutinins. The applicability for this purpose of nonhuman erythrocytes and enzyme altered cells in the test-system remains to be explored and may contribute to further differentiation.

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SUMMARY

1. Thirty-one strains representing eight species and four genera of the molluscan family Planorbidae were surveyed for the presence of hemagglutinins and hemolysins. Extracts prepared from albumen-glands and egg-masses, as well as hemolymph, were assayed by a micro-hemagglutination technique in which human erythrocytes were used as receptors.

2. Hemagglutinins and hemolysins for human erythrocytes were not uniformily present in all members of the family and detection of these substances depended, in part, on the material tested.

3. Neither heating to 56° C, dialysis, storage at −20° C for up to a year, nor repeated freezing and thawing appeared to effect the agglutinating activity of egg or albumen-gland extracts.

4. Inhibition of the agglutinating activity of *Helisoma* hemolymph could be accomplished with N-Acetyl-D-Galactosamine, but this sugar had no effect on the activity of *Biomphalaria* agglutinins.

5. There is evidence to suggest that the source and nature of agglutinins from *Helisoma* and *Biomphalaria* species are different.

6. Lectins may be of some value in characterizing snail populations and as an aid in the taxonomic discrimination of species.

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METABOLIC RELATIONSHIPS BETWEEN GREEN HYDRA AND ITS SYMBIOTIC ALGAE

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Green hydra are characterized by the presence of symbiotic, green algae in their digestive cells. These *Chlorella*-like (= zoochlorellae) symbionts have been shown to be photosynthetically active (Lenhoff and Muscatine, 1963) to facilitate the survival of the host under starvation conditions (Muscatine and Lenhoff, 1965) and to release carbohydrate (Muscatine, 1965). Symbiotic algae that have been isolated from the host and examined *in vitro* at low pH, release up to 85% of their photosynthetically-fixed ^{14}C as maltose (Muscatine, 1965). While the aforementioned studies show that algal symbionts translocate products to the host and that large quantites of carbohydrates are potentially available, no measurements have been made reporting how much material is actually translocated and utilized in the intact association. In this work the metabolic requirements of green hydra under various conditions are estimated, and the major class of metabolites being burned are deduced. Moreover, the caloric demands of the association are compared with the calories potentially available from the nutrition provided by exogenous food and available from the symbiotic algae.

The analysis consisted of measuring the respiration rates of green hydra maintained under various feeding and light regimes. From the respiration rates, respiratory quotients were calcaulated and compared. The respiratory quotient (RQ) is defined as the ratio of carbon dioxide produced to oxygen consumed during respiration (Kleiber, 1965). This ratio, which varies from 0.7 to 1.0 is indicative of the kind of food being oxidized (fat or carbohydrate) and can be used to calculate the caloric expenditure of an organism and the percentage of energy derived from carbohydrate and fat catabolism. The theoretical basis of respiratory quotients and caloric estimation (called indirect calorimetry) is a standard topic in most physiology texts (see Wilson, 1972) and is discussed at length by Kleiber (1961). The results of these experiments were compared to determinations made using aposymbiotic (algae-free) hydra.

MATERIALS AND METHODS

Experimental animals

Green and aposymbiotic (= algae-free) hydra were grown and maintained in M solution according to the methods of Lenhoff and Brown (1970). The animals were originally a gift of Dr. L. Muscatine and, lacking specific taxonomic designation, are referred to as the English strain of green hydra. These animals are distinguished from other green hydra in this laboratory by their larger size, and nematocyst dimensions (Pardy, 1976a). Aposymbiotic hydra were obtained from mass culture of algae-free clones prepared originally by bleaching green animals (Pardy, 1976b). In practice, adult green animals were exposed continuously to light (620 watts/m^2) provided by a General Electric reflector flood lamp while in the presence of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea, K and K Laboratories, Plainview, N.Y.] at 10^{-6} M prepared in M solution. During this treatment the animals were maintained unfed at 15 ± 1° C. Bleached animals resulting from this procedure were reared through several algae-free generations prior to their use in experiments. All experimental cultures were maintained in a photoperiod incubator at 18 ± 1° C with a 12-hour light/dark cycle. Illumination was provided by six 20-watt, cool-white fluorescent tubes. In some instances animals were maintained in the dark for five days prior to experimentation.

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Manometry

Gas uptake by hydra was determined using a Roger Gilmont, differential microcapillary manometer as described by Peterson, Freund, and Gilmont (1967). Uptake measurements were made at 20° C by partial immersion of the manometric apparatus in a constant temperature water bath. In practice 0.2 ml of wet packed hydra (130–200 animals, Pardy and Dieckmann, 1975), adjusted to a volume of 2 ml with M solution, were transferred to a Warburg reaction vessel. In experiments measuring total oxygen uptake, twelve drops of 10% KOH were added to the center well of the Warburg flasks to absorb the CO_2 evolved by the respiring hydra. The center wells were provided with filter paper wicks to facilitate rapid CO_2 absorption. Following a 30–45 minute equilibration period, readings of oxygen uptake were taken every five minutes for periods up to 90 minutes. The number of microliters of oxygen taken up in each of four or six flasks was regressed against time using the method of least squares. From the slopes of the regression lines the rate of oxygen uptake was calculated. Following correction of the rates for the protein content of the flasks (described below) the average respiration rate per milligram of protein per hour was computed [$= \mu l\ O_2/(mg \cdot hr)$] for the flasks. To avoid the complications of algal photosynthesis and concomitant release of oxygen in green hydra (Pardy and Dieckmann, 1975), all manometric measurements were made in the dark, provided by wrapping the reaction vessels with aluminum foil.

Oxygen uptake measurements were made on green hydra that were fed every 24 hours or unfed (= fasted). These animals were either maintained under a 12L:12D photoperiod or kept in constant darkness. Aposymbiotic animals were fed every 24 hours or fasted and maintained under a 12L:12D photoperiod. In all cases, the animals were maintained on an experimental regimen at least five days prior to experimentation and were fasted 24 hours before respiration measurements were made.

Protein determination

At the end of each experiment, the animals were carefully removed from each flask and analyzed for protein content by the method of Lowry, Rosebrough, Farr and Randall (1951). Animals were homogenized using a tissue grinder and motorized pestle. Symbiotic algae, which are unaffected by homogenization, were separated from the host tissue by low speed centrifugation ($50 \times g$). Protein analyses were then performed on the algae-free host homogenate.

Determination of respiratory quotient (RQ)

The respiratory quotient was determined experimentally by measuring the rates of oxygen uptake in the presence of the CO_2 absorbant (10% KOH placed in the center wells) and, in the absence of the CO_2 absorbant, according to the methods discussed by Umbriet, Burris and Stauffer (1964). After determining the oxygen consumption rate the animals were removed from the reaction vessels and resuspended in fresh M solution in flasks with no KOH in the center wells. Following a 45 min equilibration period, readings were taken at five min intervals for periods of up to one hour. The rate of carbon dioxide given off during respiration was taken to be the difference between the rate of oxygen uptake measured in the presence of KOH and that measured with no KOH. In these experiments RQ = (rate of CO_2 evolution)/rate of O_2 consumption) = (rate of O_2 consumption + KOH) − (rate of O_2 consumption − KOH)/(rate of O_2 consumption + KOH). The caloric equivalence of these experimentally determined RQs was determined by comparing them to the values given in standard tables by Richardson (1929).

Growth rates

In these experiments respiration rates were measured as a function of various feeding/light conditions. To assess the growth of the animals under these regimes, replicate groups of animals were maintained in 9 cm plastic petri dishes under identical conditions as the experimental hydra. Daily for seven days, the number of hydranths in each dish were counted and the growth rates and population doubling times were calculated using the method of Loomis and Lenhoff (1956).

RESULTS

When RQ values are determined for higher animals, such as vertebrates, respiration rates are corrected for protein catabolism by measuring the amount of urea or uric acid nitrogen excreted during an experimental period. The degree of protein catabolism in hydra was estimated by measuring the decrease in protein in populations of aposymbiotic hydra over a period of eleven days during which the animals were not fed. Starting with populations that had 1.3 mg of protein on day 1, a decrease of 0.5 mg protein (determined as discussed under Materials and Methods) at day 11 was detected. The catabolism of this amount of protein would require approximately 1.24×10^{-2} μl of oxygen per mg protein per day if the measured decrease in protein was due to catabolic losses alone and not due to tissue sloughing as described by Campbell (1967, 1974). Under the conditions of these experiments, this amount was judged to be insignificant and corrections of the respiration data for protein catabolism were not made.

The rates of oxygen uptake of the hydra under various conditions are shown in Table I, as are the growth rates and doubling times of the animals. The green

TABLE I

Rates of oxygen uptake and CO_2 evolution by green and aposymbiotic hydra as determined with and without absorption of respired CO_2 (+ or −KOH) under various conditions. Data are expressed as mean ± the standard deviation and $n = 4$ for all except fed light where $n = 6$. Growth rate constant (K) and population doubling time in days (d.t.) is given for each experimental group of animals.

Hydra and experimental conditions	Oxygen uptake µl O_2/(mg·hr)		CO_2 given off µl CO_2/(mg·hr)
	+KOH	−KOH	
Green hydra			
Fed light (K = 0.187, d.t. = 3.7)	55.2 ± 13.7	7.7 ± 2.4	47.5
Fed dark (K = 0.187, d.t. = 3.7)	20.5 ± 9.9	6.3 ± 6.0	14.2
Fasted light (K = 0.020, d.t. = 34.6)	21.2 ± 7.2	1.7 ± 1.6	19.5
Fasted dark (K = 0, d.t. = 0)	17.5 ± 5.6	5.1 ± 3.5	12.4
Aposymbiotic hydra			
Fed (K = 0.17, d.t. = 3.9)	17.5 ± 2.8	5.4 ± 1.7	12.1
Fasted (K = 0.03, d.t. = 21.6)	18.7 ± 2.7	4.1 ± 1.7	14.6

animals maintained under a 24 hour feeding regimen with light exhibited an uptake rate of 55.2 ± 13.7 µl O_2/(mg·hr). This rate was over twice that of animals, aposymbiotic or green, maintained under other experimental conditions, although the growth of these animals did not appear significantly different from the other fed animals (Table I). An analysis of variance showed that the rates of oxygen uptake for the green hydra (fed-dark, fasted-light, fasted-dark) and aposymbiotic hydra (fed or starved) were not significantly different ($F = 0.300$, $P > 0.75$). When the rate for the fed-light experiment (green animals) was included in the analysis, $F = 13$ and $P < 0.001$. In subsequent calculations the rate of oxygen uptake was taken to be the average of all the rates [19.1 µl O_2/(mg·hr)] excepting that determined for the green animals maintained on the fed-light regimen.

An analysis of variance performed on rates of oxygen uptake without KOH by hydra maintained under various conditions (Table I) showed no significant difference ($F = 0.616$, $P > 0.50$) between green hydra (fed-light, fed-dark, fasted-dark) or aposymbiotic hydra (fed, fasted). When the values for the fasted-light experiments were included, $F = 1.584$, $0.25 > P > 0.10$. In further calculations the rate of oxygen uptake in the absence of KOH was taken to be the average of the experiments above [5.5 µl O_2/(mg·hr)] exclusive of the rate for the green, fasted-light animals. The amount of CO_2 given off during the various experiments is shown in Table I. Green hydra maintained under a 24 hour feeding regimen with light exhibited a rate of CO_2 evolution over twice that determined for the other experiments.

Lenhoff (1965) determined the protein content for single specimens of *Hydra viridis* to be 6.32 µg. Using this factor and assuming that the animals used in the

Table II

Comparison of oxygen consumption, RQ, energy expenditure and amounts of fat and carbohydrate consumed by hydra under various experimental conditions.

Hydra and experimental conditions	Oxygen consumption μl/day per hydra	RQ	Number and percent of calories expended per day		Total calories per day	Estimated amount of food (μg) catabolized per hydra per day	
			Fat	Carbohydrate		Fat	Carbohydrate
Green hydra Fed-dark Fasted-dark Aposymbiotic hydra Fed Fasted	5.7	0.725	0.0305 (95.2%)	0.0015 (4.8%)	0.0320	3.0	3.7
Green hydra Fed-light	14.4	0.862	0.0320 (45.9%)	0.0302 (54.1%)	0.0702	3.0	9.5
Fasted-light	5.7	0.970	0.0072 (25.9%)	0.0208 (74.1%)	0.0280	0.8	5.2

present experiments were about twice the size of *H. viridis* (based on length measurements), it was estimated that their protein content was about 12.6 μg per hydra. This value was used in converting the data in Table I, which are expressed as μl O_2/mg protein·hr, to μl O_2/hydra·hr. Since the hydra are fed only once in 24 hours and growth rates and doubling times are generally expressed in days, the data were converted to μl O_2/hydra·day in constructing Table II. From Table II, it can be seen that green hydra maintained under fed-light conditions exhibited an RQ of 0.862 *vs.* 0.920 for green animals fasted with light. The other experimental conditions and animals yielded an RQ of 0.725. Using these data and values presented in standard tables (Richardson, 1929) the calories expended per day per hydra and the amount of calories derived from fat and carbohydrate catabolism were calculated. Finally, from the caloric values, the numbers of grams of fat and carbohydrate burned per day were calculated using the relationship of 9.5 kilocalories per gram of fat and 4.0 kilocalories per gram of carbohydrate (Kleiber, 1965). The results of these calculations are shown in Table II from which the following points emerge. First, fed green hydra maintained in the light consume over twice the amount of oxygen and expend over twice the amount of energy as the animals in the other experiments and by comparison, catabolize a relatively large amount of carbohydrate. These animals catabolize approximately the same amount of fat (3.3 μg) as the other experimental animals with the exception of green hydra starved in the light (0.8 μg). Secondly, green hydra starved with light expend approximately the same amount of energy (0.0280 cal/day) as the green hydra maintained in the dark (fed or fasted) and the aposymbiotic animals (0.0320 cal/day). By contrast the green hydra fasted in the light appear to derive 74% (5.2 μg) of their energy from carbohydrate *vs.* 4.8% (3.7 μg) for the other animals.

DISCUSSION

Values for the respiratory quotient vary typically from 0.7, for fat catabolism, to 1.0 for pure carbohydrate catabolism with intermediate values indicating catabolism of mixed amounts of fat and carbohydrate (Kleiber, 1965). In these experiments three different RQ values were obtained which varied according to the regimen under which the hydra were maintained. An RQ of 0.725 (Table II) was determined for fed or fasted aposymbiotic animals and for green animals maintained in the dark. Under these conditions, the hydra expended energy at the rate of 0.032 calories per day.

According to Slobodkin and Richman (1961) an individual *Artemia* nauplius weighs about 1.48 μg and has a caloric content of 0.0091 calories. Urbani (1959) measured the amount of fat and carbohydrate in *Artemia* nauplii and reported values of 0.22 μg per nauplius for fat and 0.347 μg per nauplius for carbohydrate. These amounts correspond to 0.0021 calories per nauplius for fat and 0.0013 calories per nauplius for carbohydrate. From Table II it can be seen that a hydra which exhibits an RQ of 0.725 and which consumes 5.7 μl O_2/day requires at least 3.0 μg of fat and 3.7 μg of carbohydrate. Hydra fed in these experiments ate between 8 and 12 nauplii per hydra per day and hence ingested 1.77 μg to 2.66 μg of fat (representing 0.0168 to 0.0252 calories) and 2.77 μg to 4.16 μg of carbohydrate (representing 0.0118 to 0.166 calories). The assimilation efficiency of the carbohydrate and fat components of ingested brine shrimp nauplii are not known, hence estimates are necessarily provisional. However, it is clear from these calculations that the amount of fat and carbohydrate required by fed hydra as deduced from our metabolic data and that which is potentially available from *Artemia* are in fairly good agreement. Fed animals, however, appear not to catabolize fat and carbohydrate in proportion to the amounts present in their food but seem to oxidize fat preferentially. The ratio of carbohydrate to fat in a nauplius is about 1.56, whereas the ratio of carbohydrate to fat metabolized by the hydra is approximately 0.12. Moreover, it is obvious that fed animals obtain more carbohydrate from their food than necessary to account for the observed RQ of 0.82. It is possible that this excess carbohydrate is not catabolized but rather is stockpiled as glycogen or utilized in the synthesis of other molecules without undergoing oxidation.

Fat metabolism was also indicated in fasted hydra—a condition which is obtained in higher animals (Kleiber, 1965). Kleiber (1965) reports that the caloric expenditure for fasting animals is taken to be 4.7 kcal per liter oxygen consumed. Using this factor, a value of 0.0267 calories per hydra per day is obtained which approximates the values (0.0320 calories) that were derived from the respiratory data.

Fed green hydra maintained in the light exhibited a higher respiratory rate (Tables I and II) than animals under any other condition. These animals exhibited an RQ of 0.862, indicating a mixed metabolism with a proportionate increase in carbohydrate catabolism. The source of this carbohydrate must be the algal symbionts for two reasons. First, significant carbohydrate metabolism is observed only in green hydra maintained in the light (Table II). Secondly, a maximum of 4.06 μg of carbohydrate is potentially available from hydra's food *vs.* the 9.5 μg predicted necessary for the estimated RQ of 0.862 at the oxygen consumption of 14.4 μl/day per hydra exhibited by these fed animals. The increased oxygen consumption measured in these hydra (Tables I, II) probably results from the catabolism of carbohydrates by the symbionts. Pardy (1974) has shown that green hydra symbionts do not reproduce when the host is starved with or without light. Conversely, symbiotic algae increase logarithmically, as do the hosts when the hydra are fed and maintained in the light (Pardy, 1974). The animals in the fed-light experiment were from populations growing logarithmically at a rate constant of 0.18 (Table I). Hence it is hypothesized that the observed enhanced oxygen consumption and caloric expenditure is most likely a function of the increased metabolic and biosynthetic activity associated with symbiont reproduction. Table I shows that the animal constituent of the symbiosis under these growing conditions requires the same amount of fat as predicted for the aposymbiotic hydra and green hydra maintained in the dark (3 μg), a quantity potentially available in the animal's diet of shrimp nauplii.

Fasted green hydra maintained in the light exhibit an RQ of 0.970 and an oxygen consumption rate of 5.7 μl/day. These animals show a very low growth rate constant (Table I). It is under these conditions that symbiotic hydra demonstrate enhanced survival when compared with aposymbiotic hydra (Muscatine and Lenhoff, 1965). The RQ of 0.97 derived from these data results from a decrease in the amount of fat catabolized with a concomittant increase in carbohydrate oxidation. The caloric expenditure of 0.0280 is within 88% of the estimate for fed animals and is derived from the catabolism of at least 5.2 μg of carbohydrate. The source of this carbohydrate, in view of the fact that the animals were receiving no exogenous food, must be the photosynthetic symbionts. The algae, under conditions of light and host starvation, do not reproduce (Pardy, 1973) but are photosynthetically active. The nutrients produced by the algae are shunted to the animal partner, where they are catabolized rather than being converted into new algal biomass. This process apparently spares the animal of its fat reserves (fat metabolism is reduced 75%) and thus the survival of the animal, and hence the system, is prolonged.

One process that was not accounted for in these experiments was the photorespiration of the algae. Many plants show a marked stimulation of nonmitochondrial respiration in the light. The measurement of this respiration is difficult as the substrates and products (CO_2, O_2) are common to the photosynthetic pathway. This difficulty was circumvented to a degree by performing respiratory measurements in the dark. A detailed discussion of photorespiration is found in Zelitch (1971) who points out that free-living *Chlorella* exhibit measurable photorespiration only under conditions of abnormally high oxygen concentrations or abnormally low carbon dioxide concentration. As little is known about the intracellular concentration of these substrates in hydra, estimates of photorespiration are not possible at this time.

These experiments support the generally held notion that symbiotic algae provide nutrition to hydra when the animals are undergoing starvation. In contrast to previous workers, the present efforts have yielded some reasonable estimates about the quantities of foodstuffs and energy involved. We offer the RQ of 0.970 obtained under host fasting in the light as evidence that the algae supply carbohydrate to the host and that at a respiration rate of 5.7 μl/day at least 69% of the animal's energy requirements may be supplied by the symbionts.

From this work at least two predictions emerge to be tested. First, the algae in a single hydra must be capable of releasing or translocating at least 5.2 μg of carbohydrate. Secondly, algae from hydra fed and growing in the light direct most of their photosynthate to the biosynthesis of new algae material whereas algae from unfed hydra maintained in the light divert most of their photosynthate to the host. Hence algae from starving hydra should be significantly "more leaky" than algae from fed animals.

From these respiratory measurements, it is evident that under most conditions hydra (green or aposymbiotic) obtain the major proportion of their energy from fat. When green hydra undergo starvation, the symbionts provide nutrition and the animals switch from a fat to a carbohydrate metabolism. It seems reasonable to expect that as the algae become more prone to translocation, biochemical or enzymatic switching must take place within the metabolic network of the animal cells. How the host and symbiont metabolisms are modulated is not known but control may involve host feeding. When a hydra is fed, both the animals and algae (if light is present) reproduce; when the animals are starved, reproduction of both partners ceases. Starvation conditions in the host may signal cessation of algal reproduction with concomitant diversion of carbohydrate to the host. Current work in this laboratory is directed toward examining some of these possibilities.

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SUMMARY

1. Hydra that maintain algal symbionts evidenced three modes of metabolism depending upon the nutritional state of the host and the photoperiodic conditions under which the animals were maintained. Animals either fed or fasted but maintained in the dark exhibited an RQ of 0.725 indicating fat metabolism. When they were fed *Artemia* nauplii and maintained in the light, green hydra exhibited a mixed metabolism of carbohydrate and fat which gave an RQ of 0.862. Fasting green hydra, when maintained in the light, showed a pronounced carbohydrate metabolism typified by an RQ of 0.970.

2. Aposymbiotic hydra, whether fed or fasted, exhibited an RQ of 0.725 indicating a high degree of fat metabolism.

3. Symbiotic hydra which were fed and maintained in the light demonstrated a respiration rate of 14.4 μl/day per hydra, which was 2.5 times greater than animals (symbiotic or aposymbiotic) maintained under any other condition of fasting or photoperiod. It is hypothesized that the enhanced respiration observed in these animals is due to the metabolic activities of the symbiotic algae associated with reproduction and development.

4. Calculations based on respiratory measurements and indirect calorimetry suggest that hydra consume approximately 0.0320 calories per day and that symbiotic algae may supply up to 69% of the host caloric requirements *via* the translocation of approximately 5 μg of carbohydrate when the host is fasting and light is provided.

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SEASONAL PATTERNS OF GAMETOGENESIS IN A NORTH ATLANTIC BROODING ASTEROID, *LEPTASTERIAS TENERA*

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Brooding behavior has been reported in several species of sea stars, but detailed descriptions of gametogenesis and reproductive behavior are available for relatively few species of *Leptasterias.* Recent valuable contributions to the knowledge of reproductive cycles and brooding behavior in two Northeast Pacific species of *Leptasterias* have been made by Chia (1966, 1968a, b) and Menge (1975) studying *L. hexactis* from Puget Sound, and by Smith (1971) observing *L. pusilla* from Monterey Bay. Stomach brooding behavior in the arctic species, *L. groenlandica,* was described earlier by Lieberkind (1920) and Fisher (1930).

The genus *Leptasterias* is predominently an arctic-boreal group of asteriods (Fisher, 1930; Ekman, 1953). While direct development is characteristic of many arctic invertebrates among many taxonomic groups including echinoderms (Thorson, 1946, 1950), available data indicates that the more southern-occurring species of *Leptasterias,* for example *L. hexactis, L. pusilla* and *L. tenera,* have retained this mode of reproduction even though the majority of sympatric asteroids have planktotrophic development.

Leptasterias tenera, a species of the continental shelf of the Atlantic, occurs from Nova Scotia to Cape Hatteras in the cold-temperate (boreal) marine climatic zone at depths of 18 m to 150 m. This species differs ecologically from the Northeast Pacific species *L. hexactis* and *L. pusilla* in two important respects: first, it is sublittoral as opposed to intertidal; and secondly, it is predominately an inhabitant of soft bottom sediments rather than rocky shores.

Gonads of *L. tenera* collected during a one years period were examined in order to trace the cycles of gametogenesis. Comparisons were made with the available information on the reproductive patterns in *L. hexactis* and *L. pusilla,* and modifications in the various patterns were noted. To evaluate the divergence of the reproductive pattern in a brooding starfish, *L. tenera,* from that of a broadcasting species, brief comparisons were made with the reproductive system of *Asterias vulgaris* as described by Walker (1975). Evidence was sought for a correlation between gametogenesis and the feeding pattern.

An evaluation of features common to the genus *Leptasterias* suggested that the reproductive pattern associated with direct development and brooding is genetically fixed. Since *Leptasterias* apparently lacks the genetic flexibility to "return" to the more primitive broadcasting mode of reproduction, it was suggested that the inherited pattern would be subject to selective modification. Such modifications should be discernible among species occurring in divergent ecological and zoogeographic conditions.

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Materials and Methods

Samples of *L. tenera* were collected between two locations in Block Island Sound, west of Block Island (41° 11.4′ N, 71° 38.3′ W to 41° 12.1′ N., 71° 36.2′ W) at a depth of about 30 m using a modified scallop dredge. Bottom sediments in this area of Block Island Sound consist of fine to very fine sands mixed with silts and clays. Collections were made on an approximate monthly schedule from October 1971 through September 1972, with the exception of January when no sample was taken.

Within 24 hours of collection, the arms were removed and placed in Bouin's fixative. Gonads were dissected from representative samples of each size group and preserved as above. Observations on the gonads of living animals also were examined at this time. All fixed material was embedded in paraffin and sectioned at 6–10 microns. Longitudinal and transverse sections were stained with Heidenhain's iron hematoxylin and eosin. Due to difficulties encountered in sectioning the extremely yolky oocytes, it was necessary to soak the paraffin blocks in tap water for 12–24 hours prior to sectioning.

Results were based on the study of a minimum of two animals of each sex per month. All measurements were made using an ocular micrometer. The information showing the period of active feeding in *L. tenera* which is presented in Figure 1 was obtained from monthly stomach analyses. These data will be discussed fully in another paper (Hendler and Franz, in preparation).

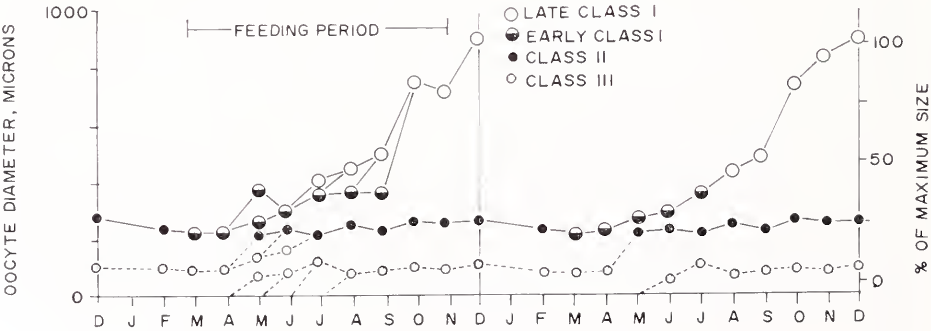


FIGURE 1. Two-year cycle of oocyte development, as determined by extrapolation from the histological examination of monthly collections during a 12 month period. The information on feeding activity is based on stomach analyses of monthly samples collected over the same period. As shown in the left panel, most oocyte growth including vitellogenesis occurred during the period of most active feeding. The right panel traces the development of a single oocyte, beginning with its first appearance in June. This small (Class III) oocyte would, most likely, become class II the following spring. Active vitellogenesis would begin the following March or April, to produce a mature (Class I) yolk-filled oocyte by November or December, a total developmental period—in this example—of 29–30 months. It is estimated that, in general, oocyte development requires a period of between 24 and 30 months.

Observations and Results

Morphology of the testes

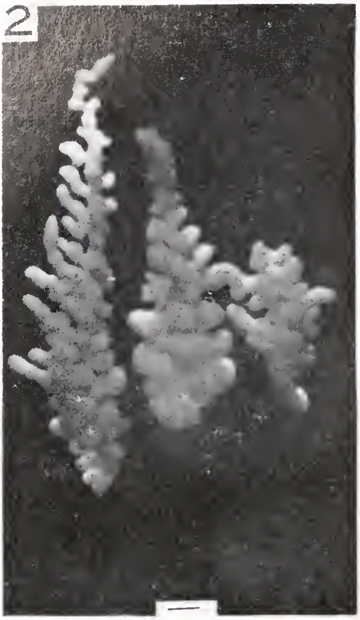
The testes in *L. tenera* exhibit considerable variability in size and shape between different individuals as well as in different arms of the same individual, and also during spermatogenesis. It is not unusual to find two testes of markedly unequal size, only one testis, or even no testis at all in a given arm. Characteristically, each arm contains two sac-like testes with finger-like projections (Fig. 2). Located ventrally in the proximal portion of the arm, they lie on either side of the ambulacral ossicles. The only attachment to the body wall is by means of the sperm duct. Each testis opens independently to the outside through a short sperm duct which leaves the coelom between the dorso-lateral and supramarginal plates and terminates ventrally in a small papilla located near the adambulacral plates. This location of the sperm duct opening is comparable to that described by Fisher (1930) in *L. groenlandica,* and by Smith (1971) in *L. pusilla.*

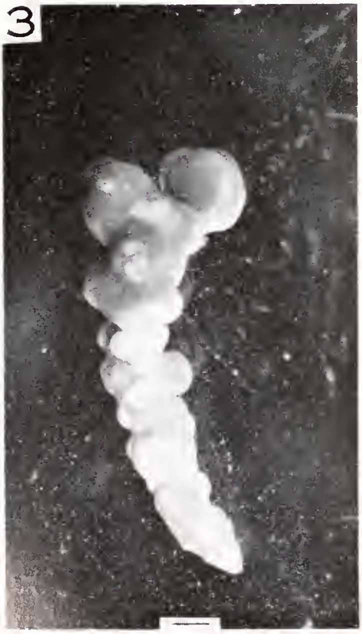
Histology of the testis wall

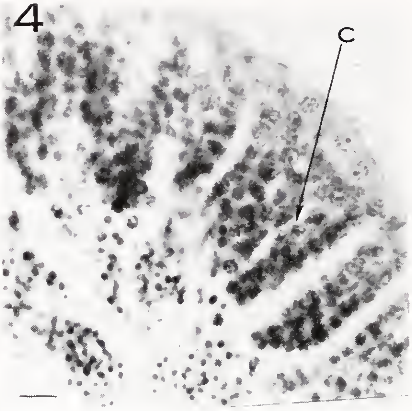
The structure of the testis wall in *L. tenera* is basically comparable to that described by Smith (1971) in *L. pusilla,* and by Walker (1974) in *Asterias vulgaris.* In *L. tenera,* the wall is composed of three parts: an outer, multilayered portion; the haemal sinus; and an inner, germinal epithelial layer which delimits the lumen. The strata which make up the outer portion include a single layer of flagellated, cuboidal, coelomic epithelial cells which become elongated and flattened during the course of gonadal enlargement, and an underlying muscle layer of both longitudinal and cross fibers which becomes thinner as the numbers of sperm increase in the lumen. Closely associated with the muscle layer is the connective tissue stratum which appears to be a syncytium of elongated, kidney-shaped nuclei in a matrix containing numerous longitudinal, radial and criss-cross fibers.

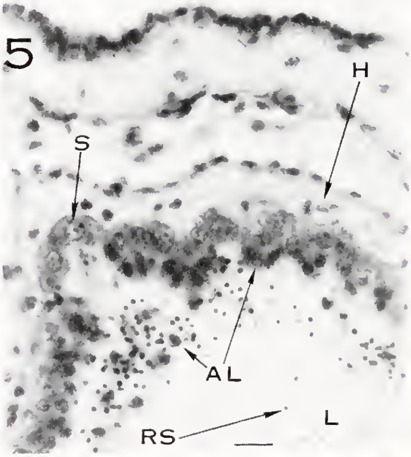
Adjacent to the connective tissue layer is the haemal sinus which is comparable to the haemal space noted by Smith (1971) in *L. pusilla* and the haemal sinus described by Walker (1974) in *Asterias vulgaris.* During the course of a year, the haemal sinus in *L. tenera* varies from an average width of about 10 microns after spawning to not more than one micron when the testis is filled with sperm. Elongated and flattened epithelial cells, widely spaced, line the sinus. Scattered within the sinus cavity are cells (called lymphocytes in this paper) lying in an amorphous, eosinophilic substance. These lymphocytes are of two types, comparable to the asteroid types described by Hyman (1955) and Endean (1966); numerous amoeboid, spindle-shaped cells, 10–16 μ; and round petaloid cells containing balloon-like swellings and very little cytoplasm, 9 μ (Fig. 6). Both Hyman and Endean considered these cells to be two phases of the same cell type, but this relationship has not been demonstrated in *L. tenera,* although both types of lymphocytes occur together.

The basal, germinal epithelium membrane is closely appressed to the haemal sinus (Fig. 5). Where infoldings of the germinal epithelium occur, the sinus also expands and fills the inpushing. A single layer of primordial germ cells appears to be present in the germinal epithelium at all times of year. Sperma-









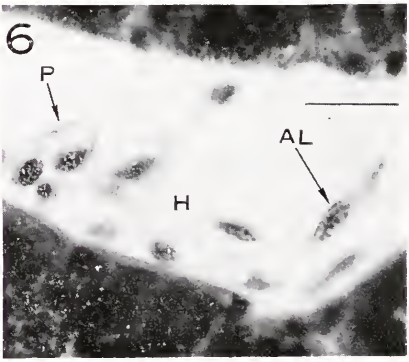


FIGURE 2. Testes from a preserved specimen taken on July 6 showing the typical appearance of the gonads. Note the many finger-like projections arising from the main axis of the testis, and the variation in size; bar is 1 mm.

FIGURE 3. Preserved ovary (November 12) shortly before spawning. Note the massive

togonia are identified by the large nucleus containing a single large nucleolus and reticular chromatin, the relatively small amount of cytoplasm, and their position close to the basal germinal membrane.

Seasonal patterns of spermatogenesis

Based on observations of preserved gonads, it is evident that shedding occurs sometime between the middle of November when the testis is expanded and the lumen is completely filled with sperm and the middle of December when all parts of the testis are greatly reduced and relatively few sperm remain in the lumen (Fig. 5). Occasionally, an otherwise spent testis may have a lobe in the pre-spawning condition indicating that shedding is not complete.

By February, the numbers of spermatogonia have increased noticeably. Differentiation into primary spermatocytes, however, is not apparent until March, which is also the time when feeding is resumed (Fig. 1). In April, primary spermatocytes are numerous and form a layer several cells thick. Spermatogenesis proceeds rapidly throughout the spring and summer months, the period of most active feeding. As the numbers of primary and secondary spermatocytes increase, they become arranged in colonnettes (Cognetti and Delavault, 1962; Smith, 1971) projecting into the lumen (Fig. 4). These formations appear comparable to the spermatogenic columns described by Walker (1976). Spermatozoa begin to break away from the tips of the colonnettes by June, and spermatids continue to mature in ever increasing numbers until the lumen is filled with masses of sperm. Colonnettes disappear by October, although large numbers of secondary spermatocytes and spermatids are still present. Just prior to sperm release, all cells complete spermatogenesis, and the germinal epithelium contains only a single layer of spermatogonia (Fig. 5).

Accessory cells

A major phagocytic function is apparently performed in the testis by the amoeboid lymphocytes. After sperm release, these cells are observed in large numbers in the haemal sinus, within the germinal epithelium squeezed between the spermatogonia, and lined up at the periphery of the lumen where they accumulate in a layer several cells thick as their numbers increase. Many lymphocytes with one or more basophilic sperm heads in the cytoplasm are present in the lumen among the relict sperm. When spermatogenesis begins in March, most of the relict sperm have disappeared. At this time, strongly basophilic, phagocytic cells filled with large basophilic granules appear in the lumen at the edge of the germinal epithelium. Dark staining, elongated cells of comparable size are squeezed between the

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protuberances, representing mature oocytes within the lumen. Light areas are immature (Class II) oocytes attached to the germinal epithelium; bar is 1 mm.

FIGURE 4. Section of testis wall (May 2) showing spermatogenesis and the formation of the colonnettes (C); bar is 10 microns.

FIGURE 5. Section of testis wall after shedding (December 21). Note the presence of relicit sperm (RS) and amoeboid lymphocytes (AL) in the lumen (L), spermatogonia (S) in the germinal epithelium, and haemal sinus (H); bar is 100 microns.

FIGURE 6. Section of testis (June 8) showing petaloid cells (P) and amoeboid lymphocytes (AL) within the haemal sinus (H); bar is 10 microns.

spermatocytes and lined up along the basal membrane. Amoeboid lymphocytes and petaloid cells are both present in the haemal sinus and in addition, cells containing deeply staining basophilic granules are frequently observed.

As the lumen fills with masses of spermatozoa, a few lymphocytes may still be seen scattered among the cells, but there is no evidence of phagocytic activity at this time. The haemal sinus becomes greatly compressed in most regions, and amoeboid and petaloid cells are concentrated in expanded areas associated with infoldings of the germinal epithelium (Fig. 6).

Morphology of the ovary

In *Leptasterias tenera,* two sac-like ovaries with many lumpy protuberances (Fig. 3) are located along the ventral floor of each arm, one on either side of the ambulacral ossicles, extending freely into the coelomic cavity. Some variation was noted in the size and shape of ovaries from different individuals of comparable size collected at the same time, as well as among ovaries in the same individual. It was not unusual to find one large and one very small ovary in the same arm, and infrequently a single ovary in a ray. Variability was less prevalent, however, than in the testes. The ovaries range from an average length of 2.5 mm soon after spawning to a maximum of 6.5 mm just prior to oviposition. Since the average length of an arm in mature specimens is about 20 mm, the ovaries, even at their maximum size, occupy less than twenty per cent of the coelomic cavity.

A single oviduct leaves the gonad on the interradial side 0.5–1.0 mm from its proximal end, extends for about 0.5 mm straight to the lateral wall of the arm and passes to the outside through an opening between the dorso-lateral and supramarginal plates III and IV. After the duct leaves the body wall, it immediately makes a right angle turn and extends under the dermis toward the mouth for 1.0–1.5 mm to open through a small papilla which lies in close proximity to the corresponding papilla of the adjacent arm. These openings are located at the level either of the fifth and sixth adambulacral spines or the sixth and seventh spines. A comparable structure and location for the oviduct openings were described by Lieberkind (1920) in *L. groenlandica,* and by Fisher (1930) in *L. groenlandica* and *L. beringensis.* There are no actino-lateral or inferomarginal spines in this region (designated the interradial platform in this paper), but one or two straight pedicellariae are usually present near the openings.

Histology of the ovarian wall

The basic histological structures of the ovarian wall in *L. tenera* are comparable in most respects to those described above in the testes. The ovarian wall however, is thicker, the connective tissue layer contains more fibers, and the haemal sinus is narrower and without amoeboid or petaloid cells. Whereas in the testis a layer of spermatogonia is present in the germinal epithelium along the basal membrane throughout the year (Figs. 4, 5), the germinal epithelium of the ovary is made up of two types of cells: first, germ cells, represented by scattered oogonia which usually appear in small clusters of a few cells and developing oocytes; and secondly, an almost continuous layer of small, numerous accessory cells which become either follicle cells or amoeboid lymphocytes. Chia (1968a) and Smith

(1971) described a similar construction of the germinal epithelium. When the oogonia differentiate into primary oocytes, they are surrounded by follicle cells and normally remain in the germinal layer, in close proximity to the basal membrane, during the course of growth and development (Figs. 7, 8, 9).

The lumen of the ovary is large and expanded at all times of the year, regardless of the number or size of the oocytes. Although the lumen is reduced in diameter following oviposition, it does not collapse as in the testis, and the walls are not folded since they still contain many developing oocytes.

Seasonal patterns of oogenesis and vitellogenesis

Meiosis in *L. tenera* is not completed until after the oocytes are shed and fertilized, a condition comparable to the pattern of oogenesis in other starfish (Tyler and Tyler, 1966; Chia, 1968a). Therefore, the developmental processes of the oocytes which take place in the ovary are concerned with increase in cell size, changes in chromosomes preparatory to meiotic division, formation of a large germinal vesicle and vacuolated nucleolus, and the development of quantities of yolk. Based on an analysis of development during a one year cycle, it is evident that completion of all these processes requires at least 26–30 months (Fig. 1), a time period similar to that postulated for *L. hexactis* (Chia, 1968a) and *L. pusilla* (Smith, 1971).

Although the developmental process is gradual and more or less continuous, it is convenient for ease of description to designate three developmental categories. These categories correspond, in general, to the three developmental stages specified by Smith (1971) in *L. pusilla,* but differ somewhat from the three descriptive stages indicated by Chia (1968a) in *L. hexactis.*

Class I. In this category are included the large second year oocytes (300–1000 microns) undergoing vitellogenesis from the first appearance of small yolk granules to the final stages when the yolk globules have become large (7–16 microns), round or elliptical, and fill the cytoplasm except for a small area surrounding the germinal vesicle. In living material, these oocytes change from white to yellow-orange to orange during this sequence of development.

Class II. Included in this category are the intermediate-sized oocytes (120–300 microns) which are undergoing cytoplasmic growth and development of a large germinal vesicle, as well as progressive vacuolation of the nucleolus. Cells in this group show no evidence of vitellogenesis and appear white in living material.

Class III. The youngest and smallest oocytes (20–120 microns) make up this category. These cells are distinguishable from the Class II oocytes by their large nucleus containing a small nucleolus and the small amount of cytoplasm. The ratio of nucleus to total cell size is about 0.34 in Class II oocytes and 0.55 in Class III cells. In living material, these small oocytes are transparent.

Seasonal changes in the size and development of oocytes throughout a two-year cycle in *L. tenera* are diagrammed in Figure 1. The feeding period of the Block Island Sound population has been superimposed on this cycle to indicate the relationship between feeding and oogenesis. Although the figure shows the smallest oocytes appearing in May, oogenesis may be initiated at an earlier date. Clusters of oogonia are present in some part of the ovary during all months. May through July, however, are the months of maximal recruitment of primary oocytes and growth, as shown in Figure 1.

Following the spawning of the fully yolked oocytes between the middle of November and the middle of December, the larger oocytes remaining in the ovary become Class I. They are easily distiguished from the smaller, and younger (now Class II) oocytes by their larger size, large, vacuolated nucleolus in an expanded germinal vesicle, and especially, by the presence of numerous, small basophilic granules scattered throughout their cytoplasm. Class III oocytes are not present, but clusters of oogonia occur in scattered areas of the germinal epithelium. Cells are identified as oocytes when they are surrounded by one or more layers of follicle cells joined in an irregular way to form an enclosing envelope. Large, unshed, fully-yolked oocytes along with some intermediate-sized oocytes are commonly present in the lumen in the process of being broken down by amoeboid phagocytes.

During the months when female *L. tenera* are brooding and feeding ceases (December through March), little visible change takes place in Class II oocytes except for a small increase in nuclear and cell size. Toward the end of this period, Class III oocytes can be identified. Class I oocytes have small yolk globules scattered throughout the cytoplasm and in some cells, a few larger globules are localized at the periphery, usually on the side toward the haemal sinus. Remnants of phagocytized, mature oocytes were found in all ovaries examined from animals collected during February and March.

With the resumption of active feeding in early April, numbers of oogonia increase. All classes of oocytes begin to show growth and development which continue throughout the summer months. As the oocytes enlarge, they bulge into the lumen while retaining their attachment to the basal germinal membrane by the encircling follicle cells. Oocytes appear squeezed together or flattened against the basal membrane (Fig. 7). Enlarging oocytes or growing Class III cells may push other oocytes away from part of their attachment to the membrane or even dislodge them completely.

In fresh, unfixed material, many of the degenerating oocytes found in the lumen during April and May appear orange. In as much as Class I oocytes do not attain even a yellow color until June, it is probable that much of the distintegrating material is still derived from unshed oocytes. However, many more Class I oocytes are undergoing vitellogenesis than will reach maturity, so that a reduction in their number must take place. It is reasonable to assume that at least some of the necrotic cells are current oocytes.

By September, numbers of oogonia decline and Class III oocytes are found in all regions of the ovary. Class II cells have increased the amount of cytoplasm and the size of the germinal vesicle while the nucleolus has become vacuolated. Class I oocytes, which appear orange in living material, are filled with numerous, large yolk globules throughout the cytoplasm, except in the immediate vicinity of the germinal vesicle which is now located at the periphery of the oocyte. Although these cells nearly fill the lumen (Fig. 9), they maintain their attachment to the basal membrane, but the follicle layer is reduced to a single band of flattened cells with elongate nuclei.

Active feeding declines from September to November. During this period, Class II and III oocytes grow very little. Class I cells, however, continue to enlarge as the yolk globules approximately double in size. Just prior to spawning (November–December), these oocytes have attained their maximum size of 900–1000 microns and lie free in the lumen. All trace of follicle cells has disappeared except for a few scattered, elongate nuclei occasionally observed between two very closely appressed cells. Immature oocytes present in the main body of the ovary are closely pressed and flattened against the basal membrane (Fig. 9), but they retain their rounded shape in the lobes and distal tip which are free of large, mature oocytes.

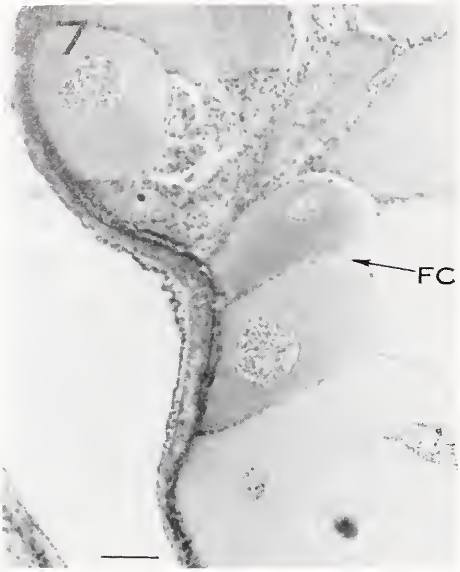
Accessory cells

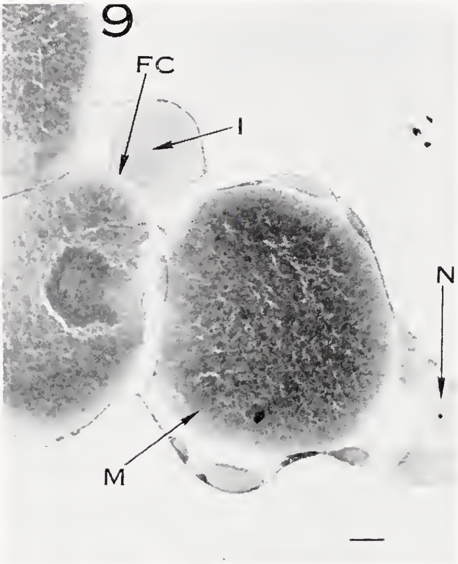
In *L. tenera*, the accessory cells in the germinal epithelium which enclose small oocytes in one or more layers of closely adhering cells, are comparable to the nurse cells observed in *L. hexactis* by Chia (1968a) and the follicle cells described in *L. pusilla* by Smith (1971). In all these species, this type of cell is presumed to play a role in the dynamics of oocyte growth. The remaining accessory cells which are not involved in follicular activity form a potential force of amoeboid phagocytes which enter disintegrating oocytes in large numbers and engulf the material. Although accessory cells are present in the germinal epithelium at all times, their numbers appear to increase markedly when degenerating oocytes are present in the lumen. Petaloid cells of undetermined origin and function were observed in the lumen of the ovary, especially during the summer months, but they were not seen in the haemal sinus at any time.

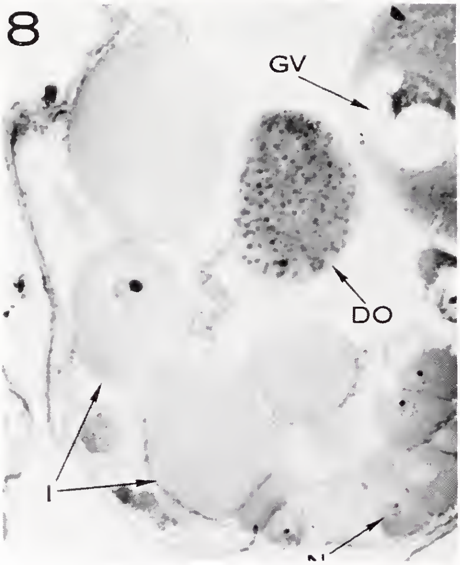
Vitelline membrane, oviposition and brooding

Both Chia (1968a, *L. hexactis*) and Smith (1971, *L. pusilla*) described localized thickenings associated with follicle cells lying outside the plasma membrane during periods of rapid growth. These thickenings gradually became the continuous, thick vitelline membrane with numerous pores or channels through which passed cytoplasmic bridges connecting follicle cells with the oocyte. Localized clusters of follicle cells around the oocyte were observed in *L. tenera*, but no evidence of their participation in the formation of a membrane was established and no direct interrelationship between follicle cells and the oocyte was observed.

At the time of spawning, a relatively small number (12–18 per gonad) of large, yolk-filled oocytes lie free in the lumen filling the main body of the ovary (Fig. 9). Oviposition undoubtedly is brought about, in part at least, by contractions of the ovarian wall forcing the oocytes into the large, open mouth of the oviduct. The walls lining the lumen of the oviduct are deeply folded which could account for expansion of the lumen to permit passage of the large oocytes. The inner margin of these folds bear great numbers of very long, stout flagella extending into the lumen. Muscle fibers are continuous from the wall of the ovary into the outer wall of the oviduct. A combination of flagellary current and muscular action could propel the oocytes to the outside. During the year, the mouth of the oviduct is small and constricted. However, the presence of amoeboid phagocytes and remnants of degenerating oocytes within the lumen of the oviduct, as well as the appearance of oocytes adjacent to the mouth being drawn into the opening of the oviduct, indicate that the flagellary current is continuously active. Since normal







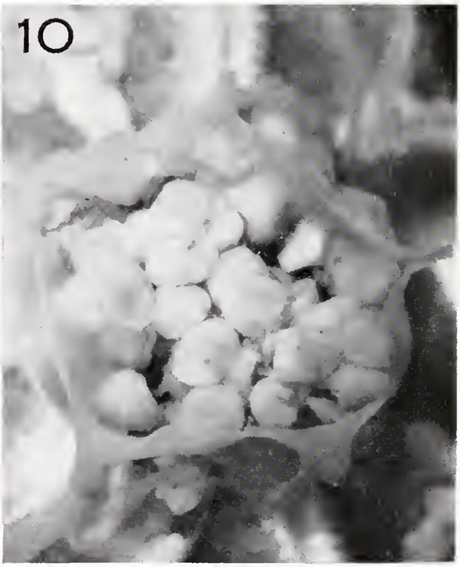


FIGURE 7. Section through the ovary (February 29) showing the attachment of developing oocytes to the germinal epithelium. Oocyte classes II and III are shown, surrounded by follicle cells (FC). Note the crowding of oocytes; bar is 50 microns.

FIGURE 8. Section through the ovary (November 2) showing detached, degenerating oocyte (DO) within the lumen. Note the position of the germinal vesicle (GV) at the periphery of the Class I oocyte. Class II and III oocytes are also shown; bar is 100 microns.

FIGURE 9. Longitudinal section through the ovary (September 1) showing three developmental classes of oocytes. Note that the yolk-filled maturing oocytes (M, Class I) lie in the lumen surrounded by stretched one-cell thick membranes of follicle cells (FC). Note that

oocytes are firmly attached to the germinal epithelial membrane, they would not be drawn into the oviduct.

The time and place of meiosis and fertilization in *L. tenera* are not known, but in a sample of more than 50 animals collected in Block Island Sound on December 21, all females contained embryos in the pyloric stomach (Fig. 10) with the exception of three individuals which had embryos in the brood chamber. Sectioned material showed that the embryos in the pyloric stomach were enclosed in a capsular membrane and had developed to the early gastrula stage, while those outside in the brood chamber were in the very late gastrula stage. Since *L. tenera* retains the embryos in the brood chamber during the later months of brooding, it is assumed that they are held in the pyloric stomach for a relatively short time, then released into the brood chamber where they adhere closely together in a compact mass and complete development in about three months.

Discussion

In general, the male reproductive system of *L. tenera* resembles that of other pentamerous species of *Leptasterias* described by Fisher (1930), as well as reproductive systems of hexamerous species which have been observed (Fisher, 1930; Menge, 1975; Smith, 1971). Some minor differences, however, were noted, especially in the reproductive cycles.

At the time of sperm release in *L. tenera,* spermatogenesis has been completed, no spermatocytes or spermatids are present in the germinal epithelium and the expanded lumen of the testis is filled with masses of mature sperm. In *L. pusilla,* on the other hand, Smith (1971) found that while the mature testis contained great numbers of sperm in the lumen at the time of shedding, colonnettes were still present, and active spermatogenesis of existing spermatocytes continued for 10–14 days after sperm release.

Smith postulated that as a result of spawning, ". . . gonial cells cease dividing until relict spermatozoa are destroyed, and then divide to produce other gonial cells and not primary spermatocytes" (Smith, 1971, p. 104). A comparable delay in the resumption of spermatogenesis was noted in *L. tenera.* Following sperm release (November/December) there was a noticeable increase in the numbers of spermatogonia, but primary spermatocytes did not appear until March. Since the period of active spermatogenesis in *L. tenera* is roughly synchronous with the most active feeding time (March–October), shown in Figure 1, it is possible that initiation of spermatogenesis is correlated with the resumption of active feeding rather than with the removal of an inhibitory factor associated with spawning or sperm destruction, especially since sperm removal continues long after spermatogenesis has begun

Following shedding of the sperm in *L. pusilla,* Smith reported that so many unshed, active, mature sperm remained in the lumen that the ". . . testes were

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they still remain attached to the germinal epithelium. Smaller Class II (I) and Class II (N) oocytes remain tightly bound to the germinal epithelium; bar is 100 microns.

FIGURE 10. Aboral dissection of female *Leptasterias tenera* showing embryos within the aboral pyloric stomach. Embryos remain within the pyloric stomach for a short time before being expelled into the brood chamber, where they are maintained for the duration of the brooding period; bar is 1 mm.

$\frac{1}{2}$ to $\frac{1}{3}$ the maximal gravid size" (Smith, 1971, p. 95). From this observation, he reasoned that the inefficient release of sperm was due to the limited contractibility of the testis wall. Muscular contraction would appear to be more efficient in *L. tenera,* since relatively few sperm remain unshed and the testes are greatly reduced in size. Although Smith did not indicate that *L. pusilla* released sperm more than once in an annual cycle, the presence of large numbers of unshed, active sperm and the continuation of spermatogenesis following spawning strongly suggest that possibility, especially since partially spawned-out animals were not found after the oocyte spawning period. A second spawning in one season would be most unlikely in *L. tenera* in as much as sperm release is nearly complete. *L. tenera* and *L. pusilla* (Smith, 1971) both produce new populations of sperm each year and unshed spermatozoa are ultimately removed by the phagocytic action of the numerous amoeboid lymphocytes.

In *L. pusilla,* Smith observed an axial core of clear, matrix-like material extending within the fingers of the colonnettes. This core was thought to be a pathway for nutrient transport to the developing germ cells. No such central matrix was noted in *L. tenera,* where the colonnettes tend to be no more than two to four cells in thickness (Fig. 4). The formation of those finger-like extensions results from sequential divisions of spermatocytes which increase the length of the colonnettes as new cells are added. There is no evidence that the developing cells are held together by more than the extended germinal epithelium.

The female reproductive cycle in *L. tenera* follows the basic pattern described for several species of *Leptasterias* (Fisher, 1930; Chia, 1968a; Smith, 1971; Menge, 1975). Production of oogonia in *L. tenera* is a continuous process. Smith (1971) on the other hand, indicated that formation of oogonia in *L. pusilla* was restricted to a five month period. Many more oogonia are produced than differentiate into the primary oocytes which then begin a two-year period of development. Of this number, only 12–18 cells per gonad become mature, fully-yolked oocytes.

During the first year of development, the primary oocyte undergoes nuclear changes resulting in the formation of a large, centrally-located germinal vesicle containing an enlarged, vacuolated nucleolus, and an increase in cytoplasmic material. More oocytes go through this stage than will complete the second year, which is devoted almost entirely to the formation and growth of yolk globules.

In *L. tenera,* as in many other species of starfish (Chia, 1968a; Smith, 1971; Walker, 1974), the basal germinal membrane is closely associated with the haemal sinus. Developing oocytes, surrounded by follicle cells, maintain a close connection with the basal germinal membrane until shortly before oviposition, when the follicle cells disappear and the mature oocytes lie free in the lumen. The location of the oocytes in close proximity to the haemal sinus throughout the developmental period suggests that at least some of the material used by the oocytes for maintenance, growth and vitellogenesis could be supplied from this channel. Chia (1968a) and Endean (1966) suggested that the haemal sinus functioned to store or transmit nutrients to the germ cells, but Smith (1971) and Ferguson (1963) discounted the importance of the haemal systems in the translocation of nutrients.

Competition for maintaining attachment to the basal germinal membrane increases as new oocytes are formed and older ones grow. Cells become tightly squeezed together, thereby reducing the extent of their attachment, and some cells become dislodged completely to lie free in the lumen (Figures 7, 8). Although the fate of such displaced cells could not be followed, it was observed that degenerating cells are characteristically found in the lumen (Fig. 8), suggesting that detachment from the basal germinal membrane may be related to subsequent breakdown. Such a "position effect", the necessity for developing oocytes to retain a critical contact with the germinal membrane, and the competition for space could account for the observed distortion and the high mortality of developing oocytes. The importance of maintaining contact with the basal germinal membrane has not been discussed in other species of *Leptasterias*.

The presence of yolk in the oocytes of *L. tenera* is recognized first in the form of small, basophilic granules scattered evenly throughout the cytoplasm of the largest oocytes remaining in the ovary after oviposition. Chia (1968a) noted similar granules which appeared at the same stage of oocyte development in *L. hexactis* and called them yolk platelets. Along with the yolk platelets, small yolk globules appear, located initially at the periphery of the cell. As the yolk globules increase in number and size and spread throughout the cytoplasm, they are in close association with large, basophilic granules which are not seen in later stages of development. At no time were these basophilic granules or yolk globules observed in association with or within the follicle cells as noted in *L. hexactis* by Chia (1968a). He suggested that yolk granules or their precursors may be synthesized in the follicle cells and later transferred to the oocytes through cytoplasmic bridges. Smith (1971) reported that in *L. pusilla* entire follicle cells may be incorporated into the cytoplasm of early second-year oocytes. In *L. tenera*, accessory cell nuclei occassionally appear to be located within the cytoplasm of a normal Class II oocyte, but serial sections of such cells show that the nuclei are actually part of the follicle envelope surrounding the oocyte.

Oocytes of all classes in *L. tenera* increase rapidly in size during the active feeding period (April–October) as indicated in Figure 1. Due to the continued growth of the yolk globules, Class I oocytes enlarge even after feeding begins to decline, and maximum size is not attained until the time of spawning. Thus, it would appear that the energy resources for vitellogenesis in *L. tenera* are derived mainly from the current feeding period rather than from reserve energy previously stored in the pyloric caeca. Chia (1968a) reported that in *L. hexactis* a rapid growth of oocytes occurred during the brooding period when the animals were not feeding and that mature oocytes stopped growing in July at a time when the animals were still feeding. The oocytes were thought to remain in a "rest period" until spawning five months later. These findings were contradicted, in part, by Menge (1975) on the basis of his studies of organ indices of several populations of *L. hexactis*. His results indicated that both ovary and storage organ (pyloric caecum) tended to increase in weight during the feeding period until October–January when spawning occurred. Consequently, a positive correlation between ovary growth and energy storage was evident up to a point and was then followed by a leveling-off period in the ovary and decline in the caecum. A preliminary examination of the relationship between annual growth curves of ovaries and pyloric caeca in *L. tenera* indicated a similar positive correlation which in this case continued until the time of spawning (Ander, personal communication).

The major role of the amoeboid, phagocytic, accessory cells is clearly the removal of disintegrating oocytes. While breakdown of oocytes may occur at any time of the year, more degenerating cells were observed late in the reproductive cycle, and following oviposition when relict ova were in the lumen. Smith (1971) suggested that phagocytic follicle cells in *L. pusilla* were instrumental in rapid and selective destruction of second-year oocytes and that nutrient material from the disintegrating oocytes was immediately made available to the remaining oocytes for completion of development. He further postulated that this recycling of nutrients constituted a ". . . mechanism for adjusting the balance between exogenous resources and the number of oocytes such that the maximum number of oocytes can complete maturation" (Smith, 1971, p. 127). Such a mechanism, if it exists would indeed be a noteworthy example of an intraspecific mechanism for density-dependent control of population density. Unfortunately, evidence was not obtained from *L. tenera* to support these hypotheses. The material, however, did indicate that breakdown of oocytes was not a rapid process, as suggested by Smith. Remnants of large, orange, yolk-containing oocytes in varying degrees of phagocytic destruction were still present in the lumen as late as April, a time when large yolk globules have not yet developed in Class I oocytes. While the energy resources presented by disintegrating oocytes may be utilized eventually by the amoeboid accessory cells, and perhaps by other cells within the ovary, there is no evidence that phagocytosis of oocytes in *L. tenera* acts as a regulatory mechanism for oocyte development.

In the description of stomach brooding in *L. groenlandica* (Lieberkind, 1920; Fisher, 1930; Hyman, 1955), the embryos were held in the cardiac portion of the stomach throughout the brooding period. In *L. tenera*, however, the embryos are retained in the pyloric stomach (Fig. 10) for a relatively short time. This difference in the site of stomach brooding is correlated with the stomach structure of the two species. The cardiac stomach of *L. groenlandica* is large and lobed, and the pyloric stomach is small (Fisher, 1930). In *L. tenera*, the pyloric stomach is greatly enlarged, while the cardiac portion is short and unlobed. The temporary retention time for the embryos within the pyloric stomach in *L. tenera* may be associated with the greater difficulty of obtaining oxygen for the growing embryos than would be experienced in the brood chamber, or even in the cardiac stomach as in *L. groenlandica.*

A comparison of the reproductive biology of *L. tenera* with the available information on other brooding species of *Leptasterias* reveals a general reproductive pattern which is especially apparent in females and may be associated, either directly or indirectly, with the brooding habit. The males show little important variation from the general asteroid pattern. The ovaries of *Lepasterias* tend to be small in relation to the size of the arms and consist of a main rachis (Walker, 1974) with small outpushings which may represent vestigial acini (Fig. 3). In some broadcasting species, such as *Asterias vulgaris* (Walker, 1974) and *Pisaster ochraceous* (Menge, 1975), ovaries are large with major acini. Large numbers of primary oocytes are produced, of which very few complete maturation. "Overproduction" of primary oocytes may represent a retention of the ancestral, oogenetic pattern characteristic of broadcasting sea stars. Rather than reduce the number of oocytes produced, a selective process within the ovary provides that only a limited number of large, fully yolked ova are spawned. In order to produce this yolk-laden oocyte, a growth period of at least two years is required, in contrast to the annual cycle characteristic of broadcasting sea stars. The rate at which vitellogenesis may be accomplished must be limited by the rate at which yolk materials or precursors can be transferred to the cytoplasm of the oocyte. Since the energy which ultimately appears as yolk is derived from food organisms, it is not suprising that the vitellogenic phase is closely associated with the feeding period. Cellular mechanisms exist for the removal and possible recycling of unsuccessful oocytes as well as unshed gametes. Such a cellular disposal mechanism would seem to be a necessity in view of the large number of oocytes which fail to survive and sperm which remain in the testes. In the ovary, cells of the germinal epithelium which develop into accessory cells become amoeboid phagocytes and break down disintegrating oocytes. The removal of relict sperm from the testes is accomplished by the phagocytic activity of amoeboid lymphocytes. It is interesting that the utilization of amoeboid lymphocytes appears to be a general phagocytic mechanism in many invertebrates, whereas the cellular phagocytic mechanism involving amoeboid accessory cells is unique to the ovary in female *Leptasterias.* Some period of parental care or brooding has been found in all species of *Leptasterias* which have been studied. Associated with the brooding pattern is the small number of yolked eggs which undergo direct development (Lieberkind, 1920; Fisher, 1930; Chia, 1955, 1968a; and Smith, 1971). A single oviduct from each gonad which opens to the outside through a ventrally located, interradial papilla is characteristic of all species of *Leptasterias* for which observations have been made. This position contrasts with the multiply-pored, dorsally located openings of the oviduct in the broadcasting starfish, *Asterias vulgaris,* described by Walker (1975).

Assuming that the above traits are genetically fixed in the reproductive strategy of the genus *Leptasterias,* natural selection can and will effect modifications upon this strategy in response to local conditions. This would be true even if the observed similarities resulted from evolutionary convergence. Although the number of species of *Leptasterias* already studied in detail is very small, certain habitat-related modifications are discernible. The number and size of oocytes produced vary significantly among species. Brood protection varies in length of time the embryos remain in the brood chamber as well as in the presence, absence and duration of the stomach phase. The correlation and timing of the reproductive cycle in relation to the feeding period show variations which are probably related to certain specific ecological factors such as feeding specialization and environmental stability.

Until further information is available for more species of *Leptasterias,* general conclusions regarding the importance of ecological factors as modifiers of the reproductive cycle in *Leptasterias* must be tentative.

SUMMARY

The reproductive system of *Leptasterias tenera,* a five-rayed, North Atlantic sea star, was studied throughout an annual cycle. In general, the gross anatomy and histological structure of the gonads were comparable to descriptions for other species of *Leptasterias* (*L. hexactis, L. pusilla, L. groenlandica*). Gametogenesis in

L. tenera followed the characteristic pattern of brooding *Leptasterias,* but certain specific variations were noted.

In *L. tenera,* spermatogenesis was completed prior to shedding, whereas in *L. pusilla,* differentiation of spermatocytes continued for at least 14 days after sperm release. Relict sperm remaining in the testis following shedding in both species were ultimately removed by phagocytic action of amoeboid lymphocytes. Two types of lymphocytes, amoeboid lymphocytes and petaloid cells, were present in the haemal sinus of male *L. tenera.*

In the female reproductive cycle of *L. tenera,* developing oocytes remained close to the basal germinal membrane and haemal sinus throughout the entire period of growth and vitellogenesis, unless squeezed out due to crowding. It was suggested that oocytes which were pushed into the lumen failed to complete development and subsequently were broken down by amoeboid accessory cells derived from the germinal epithelium. The need to maintain contact with the basal membrane was called the "position effect." Other accessory cells in the germinal epithelium become follicle cells.

In *L. tenera,* the timing of the period of greatest activity in both male and female reproductive cycles was positively correlated with the period of active feeding.

The time and place of fertilization and maturation are unknown in *L. tenera.* Sometime following spawning, the ova were placed in the pyloric stomach where the early stages of development occurred. The mass of embryos was then transferred to the brood chamber where development was completed in about three months.

Certain features of the brooding mode of reproduction apparently constitute a genetically fixed pattern within *Leptasterias.* These include a cycle of oogenesis requiring at least two years, resorption of excess oocytes, production of a small number of large, yolky ripe ova, and an extended period of brood-protection within a brood chamber. However, a comparison of reproductive patterns in three species (*L. tenera, L. hexactis* and *L. pusilla*) indicates that modifications of the genetic pattern have occurred.

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Continued from Cover Two

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The Biological Bulletin accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

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Continued on Cover Three

Vol. 153, No. 2 October, 1977

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

GENE-ENZYME VARIATION IN THREE SYMPATRIC SPECIES OF *LITTORINA*. II. THE ROSCOFF POPULATION, WITH A NOTE ON THE ORIGIN OF NORTH AMERICAN *L. LITTOREA*

Reference: *Biol. Bull.*, **153**: 255–264. (October, 1977)

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The three sympatric species of *Littorina* inhabiting the north Atlantic coasts offer an interesting system for evaluating the effect of migration on the genetic structure of species' populations. *Littorina littorea*, the large common periwinkle, inhabits the mid-intertidal region, and during its reproductive cycle it deposits fertilized eggs directly to sea where development proceeds pelagically for several weeks (Purchon, 1968). Larvae, extensively dispersed by longshore currents, then find their way ashore and metamorphose into juvenile adults. *Littorina saxatilis*, in contrast, resides in the upper-intertidal and spray zone (Haseman, 1911), and is ovoviviparous. Fertilized eggs are retained within the female and the emerging form is the juvenile adult (Purchon, 1968). In terms of larval dispersal and gene flow, then, *L. saxatilis* is quite restricted. The third species, *Littorina obtusata*, inhabits the lower-intertidal region and is a prominent grazer on *Fucus* and *Ascòphyllum* (Haseman, 1911). Female specimens of *L. obtusata* cement large egg masses onto the anchored algae, and development proceeds there to the emerging juvenile adult stage. Dispersal in this species is limited to rafting on dislodged algae.

The outcome of a genetic analysis of 15 sympatric populations of the three species collected along a 700 mile transect from Charlottetown, Prince Edward Island to the southern tip of Cape Cod, Massachusetts was reported several years ago (Berger, 1973). The major conclusion of that study, in which electrophoretic variation was followed at three nonspecific esterase loci, was that, in the two species lacking the pelagic larval stage, genetic differences could be observed at some loci between groups of populations separated by major geographic barriers (Cape Cod, and the unfavorable surface currents around Halifax). Populations within a region (for example, south of the Cape Cod Canal) were genetically similar, in general. The differences between regions, or genetic neighborhoods, came in two forms. There was either a rather sharp quantitative change in allele frequency across some boundary, or else a qualitative change occurred, where a totally new allele appeared in a region. In *Littorina littorea* the three loci were largely monomorphic in most populations; however, those rare alleles that were detected appeared at comparable frequencies throughout the range.

The results of this first survey conformed with and complemented the patterns of variation observed in other studies. For *Nassarius obsoletus* (Gooch, Smith, and Krupp, 1972), another gastropod mollusc with a lengthy pelagic larval period, and thus extensive dispersal capabilities, allele frequencies at two polymorphic loci were found to be remarkably similar among populations collected along a 1000 mile transect from Cape Cod, Massachusetts, to Beaufort, North Carolina. In contrast, allele frequencies at two polymorphic loci in *L. saxatilis* showed considerable interpopulation heterogeneity over even short distances (Snyder and Gooch, 1973).

In this report the results of several additional population genetic studies on the *Littorina* species are presented. The major work concerned an analysis and comparison of electrophoretic variation at ten to thirteen loci in natural populations of the three species collected from Cape Cod, Massachusetts, and Roscoff in Brittany, France. The purpose of this study was to determine the extent to which very long distances, across open seas, magnify the genetic differences between populations of species capable of either extensive or limited gene flow. A second study examined the temporal stability of allele frequency in several of the North American populations described in the earlier work, to determine whether the genetic differences found between populations north and south of the Cape Cod Canal were constant features of the species population structure.

Materials and Methods

Collection and preparation of specimens

Individual snails were collected at low tide and stored in perforated plastic bags containing moist seaweed until they could be processed for electrophoresis. If kept cool and aerated, the animals could remain completely viable for at least a week. In preparing the animals for electrophoresis, the shell was cracked and the soft parts (hepatopancreas and gonads mainly) were removed and homogenized on ice in two volumes of cold 10% sucrose. The homogenates were centrifuged for 10 minutes at 6000 × g, and aliquots of the supernatant were either applied directly to the gels for electrophoresis, or stored frozen at −18° C before analysis.

Electrophoresis

Procedures for 5% acrylamide gel electrophoresis, and for the localization of esterase (Est) activity, or general protein (Pt) followed the method of Hubby and Lewontin (1966). In studies utilizing 12.5% starch gel electrophoresis (Selander, Smith, Yang, Johnson, and Gentry, 1971), either of two buffer systems were used. The tris-citrate system of Nichols and Ruddle (1973) served for the analysis of lactate dehydrogenase (LDH), malate dehydrogenase (MDH), hexokinase (Hex), tetrazolium oxidase (TO), 6-phosphogluconate dehydrogenase (Pgd), glucose-6-phosphate dehydrogenase (Zw), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), amino peptidases (AP), and leucine aminopeptidase (LAP). Electrophoresis was carried out for 16 hours at 85 V. For esterase (Est), the discontinuous Poulik system was used (Selander *et al.*, 1971). The staining pro-

TABLE I

Gene enzyme variation in natural populations of Littorina saxatilis *from Roscoff and Cape Cod. Allele designations are in order of increasing relative mobility, where A represents the slowest migrating allozyme (most cathodal). Sample size (N) is noted for each locus. At six loci (TO, LDH, MDH, Hex, Pdg, Zw) the two populations were monomorphic for the same allele.*

Locus	Site	N	Allele frequency							
			A	B	C	D	E	F	G	H
Est-C	Rosc.	69	0.76	0.24						
	C. Cod	45	0.68	0.32						
LAP-1	Rosc.	72	0.96	0.04						
	C. Cod	36	1.0	0						
LAP-2	Rosc.	54	1.0	0						
	C. Cod	36	0.96	0.04						
PGI	Rosc.	71	0.01	0.24	0.62	0.11	0.01	0.01		
	C. Cod	47	0	0.14	0.03	0.83	0	0		
Est-A	Rosc.	67	0	0	0.01	0.04	0.07	0.49	0.26	0.14
	C. Cod	46	0.12	0.33	0	0	0.03	0	0.42	0.09

cedures for these various enzymes were from Selander *et al.* (1971) and Shaw and Prasad (1970).

RESULTS

The Roscoff and Cape Cod populations

Between 11 and 13 gene-enzyme systems were examined by acrylamide and starch gel electrophoresis in the three species, from single collections made in Brittany, France, and Cape Cod, Massachusetts. The results of this analysis are presented in Tables I through III.

In *L. saxatilis* (Table I) 11 enzyme loci were examined. At six of these (TO, LDH, MDH, Hex, Pgd, Zw) the two populations were found to be monomorphic, and in each case allozymes in the two populations were indistinguishable by electrophoresis. Sample sizes ranged from 40–47. At three loci (Est-C, LAP-1, LAP-2) either one or both populations were polymorphic and allele frequencies in both were quite similar. At the PGI locus the Roscoff population was found to be segregating six different alleles; however, three were at very low frequency. In the Cape Cod population only the three most common alleles were found, but at frequencies quite different from the Roscoff collection. Only at EST-A did there appear to be important qualitative genetic differences between the two populations: the Roscoff population was found to maintain six distinct alleles, and the Cape Cod population maintained five. However, only three of the total of eight alleles were shared. The average heterozygosity value calculated over the 11 loci in the Roscoff population was 0.15, and in the Cape Cod population it was 0.12.

In *L. obtusata* a total of 13 loci were examined by a combination of starch and acrylamide gel electrophoresis. At seven loci (LDH, MDH, Hex, AP-1, AP-2,

TABLE II

Gene-enzyme variation in natural populations of Littorina obtusata *from Roscoff and Cape Cod. Allele designations are in order of increasing relative mobility, where A is slowest migrating allozyme at that locus. At seven loci (LDH, MDH, Hex, AP-1, AP-2, Pt-1, LAP-2) both populations were fixed for the same allele.*

Locus	Site	N	Allele frequency						
			A	B	C	D	E	F	G
PGI	Rosc.	72	0	0.01	0.27	0	0.64	0.01	0.07
	C. Cod	9	0.35	0	0.15	0.35	0.15	0	0
Est-3	Rosc.	65	0.16	0.37	0.43	0.01	0.02		
	C. Cod	37	0	0.40	0.60	0	0		
Est-1	Rosc.	79	0.79	0.21					
	C. Cod	45	0.58	0.42					
Est-4	Rosc.	18	0.78	0.08	0.11	0.03			
	C. Cod	26	0.94	0.06	0	0			
Pt-2	Rosc.	8	0.12	0.88					
	C. Cod	23	1.00	0					
LAP-1	Rosc.	54	0.02	0.96	0.02				
	C. Cod	24	0	1.00	0				

Pt-1, LAP2; N = 16–64) both populations appeared monomorphic for the same allele. At the remaining loci (Table II), either one or both populations were polymorphic. For LAP-1 Est-4, Est-3, and Est-1 both populations maintained the same alleles at high frequency, although in three cases the Roscoff population had low frequency alleles present which were absent at Cape Cod. At the Pt-2 locus, the two populations shared alleles in common, but at Roscoff a unique allele was at high frequency. Only at PGI did there appear to be significant genetic differences between the two populations, although, even here, two of the seven total alleles are commonly shared. Values of heterozygosity in the Roscoff and Cape Cod populations were 0.17 and 0.13, respectively.

Twelve gene-enzyme systems were examined in *L. littorea* (Table III). Of these, three (AP-2, LAP-1, LDH) were monomorphic in both populations, for the same allele (N = 12–72). At two loci, Est-A and LAP-2, the two populations shared an allele in common, but this allele was always rare in Roscoff, and fixed in Cape Cod. At seven loci, the two populations were completely different genetically. Calculated values of average heterozygosity for the Roscoff and Cape Cod populations were 0.15 and 0.03, respectively.

Temporal variation

When two geographically separate populations are found to differ genetically, it becomes important to examine their temporal stability if one wishes to explain those differences by an argument involving gene flow. Table IV summaries the results of allozyme analysis on several populations taken over a number of years. The three loci studied here correspond to those described in an earlier report (Berger, 1973).

For the most part, allele frequencies in *Littorina* populations have stayed rather constant from year to year. There are several obvious exceptions, especially *L. obtusata* at Rockland, where significant differences were apparent. At the Maine sample, however, the collections made over the two years were done at different beach sites. This could account for the large variation. Nevertheless, the general qualitative and quantitative differences that were reported for the 1972 collection (the absence of *L. obtusata* Est-$1^{S'}$ allele, and *L. saxitalis* Est-$3^{S'}$ allele south of the Cape Cod Canal) still appear to be present, and apparently represent long-term features of these species' population structures.

Microgeographic variation in L. obtusata

In the Cape Cod region *L. obtusata* can range in shell color from a light yellow (almost white) to ebony. Shell colors of several intermediate hues (orange, brown) can be discerned, and the presence of distinctly striped individuals suggests

TABLE III

Gene-enzyme variation in natural populations of Littorina littorea *from Roscoff and Cape Cod. Allele designations are in order of increasing relative mobility, where A is the slowest migrating (most cathodal) allozyme at that locus. Sample size (N) is noted for each locus. At three loci (AP-2, LAP-1, LDH) both populations were fixed for the same allele.*

Locus	Site	N	Allele frequency						
			A	B	C	D	E	F	G
PGI	Rosc.	71	0.02	0.03	0.77	0.18	0	0	0
	C. Cod	62	0	0	0	0	0.01	0.02	0.97
PGM1	Rosc.	65	0	0	0.06	0.35	0.59		
	C. Cod	44	0.08	0.92	0	0	0		
PGM2	Rosc.	67	0	0.08	0.91	0.01			
	C. Cod	72	1.00	0	0	0			
AP-1	Rosc.	12	1.00	0	0				
	C. Cod	12	0	0.54	0.46				
Est-C	Rosc.	71	0.62	0.38	0				
	C. Cod	63	0	0	1.00				
AP-3	Rosc.	32	1.00	0					
	C. Cod	16	0	1.00					
MDH	Rosc.	32	1.00	0					
	C. Cod	16	0	1.00					
Est-A	Rosc.	70	0.04	0.02	0.54	0.40			
	C. Cod	63	1.00	0	0	0			
LAP-2	Rosc.	72	0.98	0.02					
	C. Cod	16	0	1.00					

TABLE IV

Four year survey of Esterase polymorphisms in three littorinid species.

Locale	Sample Size	Year	*L. obtusata*								*L. saxatilis*					*L. littorea*			
			Est-1			Est-3			Est-4		Est-3*			Est-4		Est-3*		Est-4	
			F	S	S′	F	S	O	F	S	F	S	S′	F	S	F	S	F	S
Woods Hole	22–33	1972	0.44	0.56		0.35	0.65		0.48	0.52	0.47	0.53		0.05	0.95	0.92	0.08		1.0
	24–47	1973	0.43	0.57		0.27	0.73		0	1.0	0.47	0.53			1.0	1.0			1.0
	20–41	1975	0.44	0.56		0.44	0.56		0	1.0	0.53	0.47		0.01	0.99	0.99	0.01		1.0
	16–30	1976	0.41	0.59		0.38	0.62		0	1.0	0.42	0.58		0	1.0	1.0			1.0
Manomet	23–24	1972	0.22	0.78		0.40	0.60		0.08	0.92	0.19	0.54	0.27	0.02	0.98	1.0			1.0
	16–47	1973	0.13	0.83	0.03	0.56	0.44		0.03	0.97	0.40	0.53	0.07		1.0	1.0			1.0
	37–41	1976	0.06	0.86	0.08	0.36	0.64		0	1.0	0.18	0.60	0.21		1.0	1.0			1.0
Rockland/ Bar Harbor	13–24	1972	0.37	0.63		0.31	0.69		0	1.0	0.10	0.57	0.33	0.14	0.86	1.0			1.0
	14–24	1973	0.06	0.94		0.54	0.37	0.09	0	1.0	0.18	0.59	0.23	0.12	0.88	1.0			1.0
Prince Edward Island	5–28	1972	0	0.41	0.57	0.15	0.85		0	1.0	0.51	0.34	0.15	0.01	0.99	0.92	0.08		1.0

* In 1973 high resolution electrophoresis detected heterogeneity among one of the allelic classes at each of two loci. However, this variation could not be scored unambiguously and the two variants found within the *Est-3*^{F} of *L. littorea* and *Est-3*^{S} of *L. saxatilis* are pooled.

TABLE V

Microgeographic variation of shell color and enzyme phenotype in Littorina obtusata, *based on data collected in August, 1972. Collections were made from Quisset Harbor and Gansett Point, near Woods Hole.*

Site	N	Est-1			Est-3		Shell color		
		F	S	O	F	S	N	Yellow (%)	Brown (%)
Gansett	24	0.54	0.44	0.02	0.48	0.52	35	0.06	0.89
Quisset-A	44	0.48	0.51	0.01	0.36	0.64	275	0.20	0.78
Quisset-B	20	0.55	0.43	0.02	0.39	0.61	144	0.38	0.55
Quisset-C	48	0.53	0.46	0.01	0.48	0.52	200	0.23	0.74
Quisset-D	48	0.53	0.46	0.01	0.42	0.58	125	0.16	0.83
Quisset-E	45	0.52	0.50	0	0.41	0.59	86	0.27	0.73

that the basis for shell color polymorphism is genetic. Superimposed on the color variation is an obvious polymorphism for band thickness in the striped individuals. In Manomet, for example, one finds that striped shells consist of a large number of very fine pigment bands, while at Nobska, striped shells contain only four or five broad bands of alternating color.

One striking observation made repeatedly is that the frequencies of shell color variants in the population can change dramatically over even short distances. Since it has already been shown for *L. saxatilis* that shell color probably serves as protection against predators (Heller, 1975) and, therefore, can be under strong selection, it was of interest to look for any possible nonrandom association between shell color and allozyme frequency. This association, if it existed, need not be direct; that is, the enzyme under study would not have to participate in pigment formation. Rather, such nonrandom association could arise by a form of genetic drift established when strong selection for a particular shell color genotype is, by chance, associated with one or more allozyme alleles.

In one locality, Quisset Harbor, the microgeographic cline in shell color frequency was particularly striking. As one moved from the exposed beach at Gansett to the secluded harbor at Quisset, about one-half mile, the frequency of yellow morphs increased from 6% to 38% (Table V). Snails were collected at random from six sites along the transect, and esterase allele frequencies at two loci were examined. As seen in Table V, despite the variation present with respect to shell color, allozyme frequencies were quite similar at all the sites. This observation was confirmed by carefully analyzing shell color and enzyme genotype in a large number of snails collected from Quisset Harbor, Manomet Point, and Nobska (data not shown).

DISCUSSION

The principle aim of the work on *Littorina* has been to evaluate the effect of larval dispersal on the genetic structure of gastropod species. The phenotypic markers which have been followed are allozymes, because they are easy to analyze in large numbers of individuals and can be readily and unambiguously converted into genotype and allele frequencies. Although formal genetic analyses have not been carried out on these snails, it has become customary to equate electrophoretic banding patterns with genotypes when the population contains a distribution of one-banded (homozygotes), and two- or three-banded (heterozygotes) individuals in Hardy-Weinberg proportions.

Assuming that adult specimens of *Littorina* are sessile, the obvious expectation is that any two populations of *L. littorea*, because of its pelagic larvae, should be more alike genetically than sympatric populations of *L. obtusata* or *L. saxatilis*. Furthermore, increasing distance should magnify the between-population differences in all three species. While this general pattern was observed in early studies examining gastropod populations along the North American coast (Gooch *et al.*, 1972; Berger, 1973; Snyder and Gooch, 1973), the comparison of North American and French *Littorina* shows quite the reverse. The basis for this unpredicted result, I believe, involves the probable origin of North American *L. littorea*. I will deal with this issue first, and return briefly to the general question of gene flow in turn.

The unintentional transport of animals beyond their normal geographic range has always fascinated zoogeographers. One impressive and recent case involves the introduction of the common periwinkle, *Littorina littorea,* to the rocky intertidal coast of New England. *L. littorea* was first recorded in North America at Pictou, Nova Scotia, in 1840 (Ganong, 1886). Later accounts document the subsequent and rapid southern expansion of this species' habitat range (Morse, 1880; Wells, 1965). Today *L. littorea* occurs from Labrador to Maryland and is one of the most abundant members of the intertidal mollusc community (Bequaert, 1943).

The precise origin of North American *L. littorea* has been debated for over 90 years. Ganong postulated that *L. littorea* was introduced by European colonists at the beginning of the century, and that its rapid southern expansion from Nova Scotia reflects its active dispersal capabilities (see Berger, 1973) and its success in comparable temperate habitats along the European coast. His argument is that careful malacological records of New England and the Gulf of St. Lawrence fail, entirely, to include this species, although the related species *L. palliata* (*obtusata*) and *L. rudis* (*saxitalis*) are routinely found. Moreover, no trace of *L. littorea* had been reported from any North American indian shell heap, or from any Post-Pliocene deposits of Greenland, Labrador, Canada, or New England, while shells of other *littorinid species* were common.

Clarke and Erskine (1961) have reported the discovery of several *L. littorea* shells from Micmac shell heaps near Halifax, Nova Scotia, which were determined by carbon dating to be about 700 years old. In order to explain the recent pattern of habitat expansion, Clarke and Erskine proposed that *L. littorea* was native to the Halifax area before the advent of European culture. Its failure to spread prior to the middle of the 19th century was attributed to unfavorable oceanographic factors, specifically, that the major surface currents around Halifax move eastward, out to sea, establishing a formidable barrier to the southern movement of pelagic eggs or larvae. With the advent of commercial shipping between the Maritime Provinces and southern Nova Scotia or New England, passive transport of *L. littorea* occurred, permitting colonization to begin. Further expansion was mediated by the favorable long-shore currents in the region.

A partial solution to this controversy appears to emerge from an examination of the allozyme data in Tables I–III. For *L. obtusata*, at 8 of 13 loci, both populations maintained the same allele or alleles. At the remaining five loci both populations shared alleles in common, but showed site-specific alleles as well. At no locus were the two populations completely different genetically. A similar picture emerges from an analysis of the *L. saxatilis* data. In contrast, at 7 of 12 loci examined, the two populations of *L. littorea* were completely different genetically. At the remaining 5 loci, alleles common to both populations were found. The data are in support of Clarke and Erskine's (1961) hypothesis that, indeed, *L. littorea* is probably an aboriginal species to North America, and that a very recent European origin is unlikely.

One final point of interest emerges from a calculation of average heterozygosity (Lewontin and Hubby, 1966) in these populations. This value is simply calculated by dividing the sum of all expected heterozygote frequencies by the total number of loci examined. For *L. obtusata* (*L. saxatilis*), the values for Roscoff and Cape Cod are comparable—0.17 (0.15) and 0.13 (0.13), respectively. For *L. littorea* the values for Roscoff and Cape Cod are quite different—0.15 and 0.03, respectively. This low value for Cape Cod *L. littorea* indicates a founder effect; reduced variability produced by a small initial sample of colonists. Thus, one may conclude that the rather profound genetic differences and levels of heterozygosity found between *L. littorea* from Europe and North America reflect an ancient population divergence and severe bottleneck in the North American population size. The lack of allele overlap between the two populations is, in fact, comparable to that between different species (Lewontin, 1974). It is unlikely that an entirely acceptable explanation can ever be made for the recent release of this species from Nova Scotia, since shipping along the coast of North America must have occurred prior to the nineteenth century.

Since *L. littorea* is characterized by the most extensive dispersal capabilities among the three species studied, and yet shows pronounced genetic differences between coasts, it is unlikely that significant gene flow occurs across the Atlantic for any species of *Littorina*. Thus, investigations into the effect of dispersal must be limited to studies along the Atlantic coasts. On that count, it is of importance to note that populations of all three species are characterized by temporal constancy of gene frequency. Practically, this allows one to compare geographically separate populations at any instant and evaluate the effect of space unconfounded by genetic changes which may occur over time. Certainly this interesting system of sympatric species may allow for an evaluation of gene flow on population genetic structure, internally controlled for phylogenetic similarity and gene homology.

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SUMMARY

Gene-enzyme variation was examined at ten to fifteen loci in three sympatric species of *Littorina* collected from Cape Cod, Massachusetts and Roscoff, Brittany. The North American and French populations of *L. saxatilis* and *L. obtusata* were, in general, quite similar in allele content and frequency. In contrast, the North American and French populations of *L. littorea* were genetically differentiated at a majority of their loci. This pattern of heterogeneity in the three species, along with calculated values of average heterozygosity, suggests that North American specimens of *L. littorea* are not recent colonists from Europe and have passed through a severe population bottleneck on the North American continent. Survey studies carried out over a four year period revealed a general pattern of temporal constancy in allele frequency in all three species.

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STUDIES IN THE DYNAMICS OF GENETICALLY VARIABLE POPULATIONS. II. GROWTH OF EXPERIMENTAL POPULATIONS OF *DROSOPHILA MELANOGASTER* EXPERIENCING INTENSE NATURAL SELECTION

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Although population biology originated from both population genetics and ecology, its genetical models usually neglect organismic ecology, while theoretical population dynamics largely ignores genetical variation. These two disciplines have been most successfully blended in the theory of "r and K selection" (MacArthur and Wilson, 1967; Pianka, 1970), for which the underlying genetics more recently has received theoretical investigation as "density-dependent natural selection" (Anderson, 1971; Anderson and King, 1970; Charlesworth, 1971; King and Anderson, 1971; Roughgarden, 1971; Charlesworth and Giesel, 1972b); and in the theory of "kin selection" (Hamilton, 1964; Wilson, 1975). Another, more restricted blend is "age-dependent selection" (Charlesworth, 1970, 1972; Charlesworth and Giesel, 1972a), which incorporates demographic theory into selection theory. Theoretical development of other areas remains spotty or of a largely superficial nature; that is, most theoretical population biology remains in the domain either of ecology or of population genetics.

There has been surprisingly little experimental investigation of these "hybrid" theories, though many observational data may be fit to them. Gadgil and Solbrig (1972) provided virtually the only experimental test of the theory of "r and K selection", and some of the few experimental investigations of "density dependent selection" (Birch, 1955; Bhalla and Sokal, 1964; Druger and Nickerson, 1972; Kojima and Huang, 1972; Lewontin, 1955; Lewontin and Matsuo, 1963; Moree and King, 1961; Sokal and Huber, 1963; Sokal and Karten, 1964; Sokal and Sullivan, 1963) yield ambiguous results (DeBenedictis, 1977). Elsewhere, the influence of population composition (DeBenedictis, 1978) and of variation in both population density and composition (DeBenedictis, 1977) on fitness and gene dynamics of flies in experimental populations of *Drosophila melanogaster* is described. This study reports the dual to these earlier findings: how population dynamics of these experimental populations were influenced by variation in their genetic composition.

MATERIALS AND METHODS

Experimental populations of *Drosophila melanogaster* were synthesized from flies homozygous or heterozygous for fourth chromosomes marked by the recessive mutants in repulsion linkage: either by *sparkling-poliert* (hereafter *s*) or else by *cubitus-interruptus* and *shaven-naked* (hereafter *c;* for descriptions see Lindsey and Grell, 1968). These chromosomes exhibit the Mendelian genetics of alleles of a single locus (Bundgaard and Christiansen, 1972; Prout, 1971a, b) for which homokaryotypes have a mutant phenotype and heterokaryotypes have a wild-type phenotype; Bundgaard and Christiansen (1972) detail fitness components for this system. Handling of cultures is described in DeBenedictis (1977, 1978). Briefly, flies were raised at 23°C with a 12-hr photoperiod on an agar-molasses-cornmeal medium in one-pint bottles. Contrived mixtures of virgin f_1 progeny from stock cultures became the parents of experimental cultures. After four days, parental flies were removed from experimental cultures and censused to sex and karyotype. Thereafter, the f_1 progeny of these parental flies were removed from cultures at four day sampling intervals until such time (20 days after initiation of cultures unless otherwise noted) that these progeny could have been contaminated by f_2 flies. The f_1 progeny were censused to sex and karyotype and then discarded. Discrete sampling times were adopted because they accord best with the genetical theory from which fitness was estimated and, except for constraints imposed by *Drosophila* biology, procedures for handling cultures were otherwise arbitrarily adopted.

Because selection was density-independent in this system (DeBenedictis, 1977), population density of adult flies was measured without regard to karyotype. Population growth was measured either as the total progeny obtained from all censuses of a culture, or as a finite population growth rate, λ. Although population age structure usually arises from successive births in a cohort of parents as those parents age, in the present study "age structure" arises because the offspring of a single age class of parents mature over several successive sample times. In either case, λ satisfies:

$$\lambda^{\beta+1} - \sum_{x=\alpha}^{\beta} \{B(t+x)/N(t)\} \lambda^{\beta-x} = 0$$

where α is the sample interval at which offspring first mature, β is the sample interval at which offspring last mature, $B(t+x)$ is the number of offspring obtained on the xth census after parents are removed from a culture, and $N(t)$ is the number of parents of those offspring. Total progeny and finite population growth rate were highly correlated; over 95% of the variation in $\ln(\lambda)$ could be attributed to variation in ln(total progeny/parent). The remaining variation reflects the spacing of "births" among the progeny obtained from a culture.

Wallace (1974) shows that *Drosophila* population dynamics exhibit "female dominance" (Keyfitz, 1969: chapter 13.2). Therefore, sex ratios (M/F) of progeny were also examined because the number of females in a cohort is a major determinant of that cohort's growth rate.

Five series of experimental cultures were set out from four sequential generations of stock cultures. Each experimental series had distinct goals, which are described below. In the following discussion "monomorphic" cultures produce only one karyotype of offspring, while "polymorphic" cultures yield all three karyotypes. Because population growth declined with increasing population density, null hypotheses generally were tested most effectively by analyses of covariance. Rejection level for null hypotheses was set at 0.05.

Results

Independence of the dynamics of successive generations

Experimental designs for this report assume that gene and population dynamics may be specified by measuring changes from a range of initial conditions over a single generation. Bundgaard and Christiansen (1972) and Prout (1971b) provide experimental verification that single generation adaptive value estimates predict gene dynamics of this and of similar genetic systems. Because mean size and, correlated therewith, fecundity of *Drosophila melanogaster* decreases with increasing crowding (Chiang and Hodson, 1950; Barker and Podger, 1970b), population growth potentially could be influenced by the crowding experienced by the previous generation even under otherwise uniform conditions (Shorrocks, 1970). To determine the importance of such effects, population dynamics in cultures descended from crowded or uncrowded stocks were compared. This series of cultures is hereafter called the *stocks experiment*. Stock cultures were initiated either with 25 or with 100 pairs of flies. From these stocks, cultures that would be monomorphic for *s/s*, *s/c*, *c/s*, and *c/c* karyotypes were established, in which parental density was varied from 25 to 100 pairs of flies. Although low productivity of crowded stocks precluded completion of a planned balanced experimental design, sufficient data were obtained to compare population dynamics of cultures descended from crowded *versus* uncrowded stocks by covariance analysis.

The number of surviving female parents was a better predictor of total progeny and of λ than were either total surviving parents or number of parents introduced to experimental cultures. Both total progeny and λ were exponential functions of the number of surviving female parents (Fig. 1). Neither measure, when adjusted for differences in the number of surviving female parents, was consistently influenced

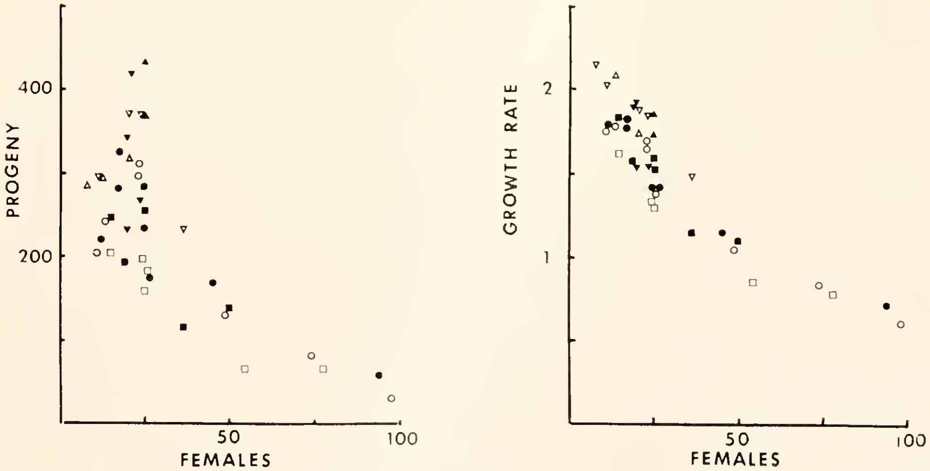

Figure 1. Total progeny (left) and finite population growth rate (right) as functions of female parental density, when parents derived from crowded (solid symbols) or from uncrowded (open symbols) stocks and cultures are monomorphic for: *s/s* (circles), *s/c* (triangles), *c/s* (inverted triangles), or *c/c* (squares).

Table I

Covariance analysis of the stocks experiment.

Source	Regression Total Progeny	 Finite growth rate
Uncrowded stocks	$P = 412 \exp(-0.0246N_t)$	$\lambda = 2.192 \exp(-0.0137N)$
Crowded stocks	$P = 386 \exp(-0.0195N_t)$	$\lambda = 2.188 \exp(-0.0132N)$
Common	$P = 399 \exp(-0.0235N_t)$	$\lambda = 2.194 \exp(-0.0135N)$
F, between slopes (d.f. = 1,35)	1.26	0.07
F, adjusted mean (d.f. = 1,36)	0.80	0.80

by the crowding experienced by stock cultures (Table I); *c/c* homokaryotypes from uncrowded stocks grew less rapidly than did other karyotypes from uncrowded stocks, while cultures begun with *c/c* females and *s/s* males from crowded stocks grew more rapidly than did the other karyotypes from crowded stocks.

The sex ratio of progeny averaged 1.07, a value not satistically different from 1, and was not influenced by the strain of fly tested, the crowding experienced by either stock cultures or by parental flies, nor did it vary consistently between censuses of progeny from a given experimental culture.

The absence of a demonstrable influence of the crowding experienced by stock cultures on population dynamics suggests that these dynamics are fundamentally Markovian in nature, and justifies use of single-generation parameter estimates to describe the dynamical behavior of this system.

Characteristics of karyotypes in isolation

Population growth parameters of the different karyotypes growing in isolation were measured in three different experiments, including the stocks experiment. A similar series of monomorphic cultures, set out from uncrowded stocks, was established to measure population dynamics of *s/s, s/c, c/s,* and *c/c* karyotypes over a broader range of initial population densities. In this series of cultures, hereafter called the *strains experiment,* parental flies were not recounted after being removed from cultures; insufficient virgin females were available to establish the full range of densities for cultures that produced heterokaryotypic offspring.

Heterokaryotypes are particularly difficult to characterize, because cultures do not remain monomorphic for a full generation. In the two experiments described above, the f_1 were either all homokaryotypes or else all heterokaryotypes, but parents of the latter were homokaryotypes (*s/s* × *c/c* or *c/c* × *s/s*). Properties of heterokaryotypic parents were measured in a small series of cultures initiated with *s/c* or *c/s* parents, set out from excess stocks of the density experiment (described below), and intended primarily to measure relative larval viability. These cultures, which constitute the *viability experiment,* were compared with cultures monomorphic for the two homokaryotypes that were established simultaneously.

Results of these three experiments were inconsistent, perhaps because different batches of medium and, sometimes, yeast were used. Population growth of all karyotypes in the viability experiment was much less vigorous than in the stocks or strains experiments. Numbers of progeny obtained from *D. melanogaster* cul-

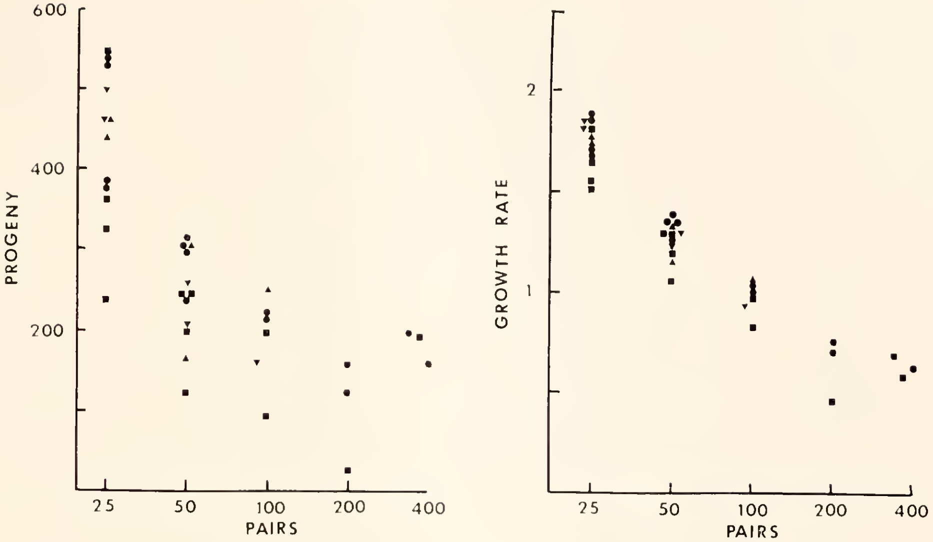

FIGURE 2. Total progeny (left) and finite population growth rate (right) as functions of the number of pairs of parental flies in the stocks experiment. Karyotypes indicated as in Figure 1.

tures commonly decline with crowding until some critical density is reached, after which increasing density may be accompanied by an increase in progeny number (Chiang and Hodson, 1950). The strains experiment spanned that critical density (Fig. 2), and regressions between progeny number or λ and log(parental density) were significantly curvilinear in this experiment only. However, even if higher density cultures in the strains experiment are ignored, its results remain distinct from the other two experiments.

In spite of these differences, two significant features were common to all experimental series. First, in each experiment, the regressions of initial density on total progeny or on λ for all karyotypes were parallel; only adjusted means (Table II) or, equivalently, carrying capacity differed. Writing these regressions as $y = a \exp\{\text{slope}\}$, the least squares estimates of the term, {slope}, common to all karyotypes are given in Table II. Secondly, within each experiment, the largest adjusted mean is statistically greater than the smallest adjusted mean; that is, some difference between the estimated carrying capacity of the several karyotypes was evident in every series. While more detailed examination of differences between adjusted means was impossible because of unequal sample sizes, no other differences were suggested in most instances (Table II). In general, *c/c* homokaryotypes tended to have the lowest carrying capacity, and heterokaryotypes are not markedly more successful than *s/s* homokaryotypes, as would be suggested by fitness differentials (DeBenedictis, 1977).

The sex ratio of progeny was not influenced by initial density of cultures but, in the stocks and in the strains experiments, it differed between strains. In the strains experiment, cultures producing *c/s* offspring only were slightly deficient in

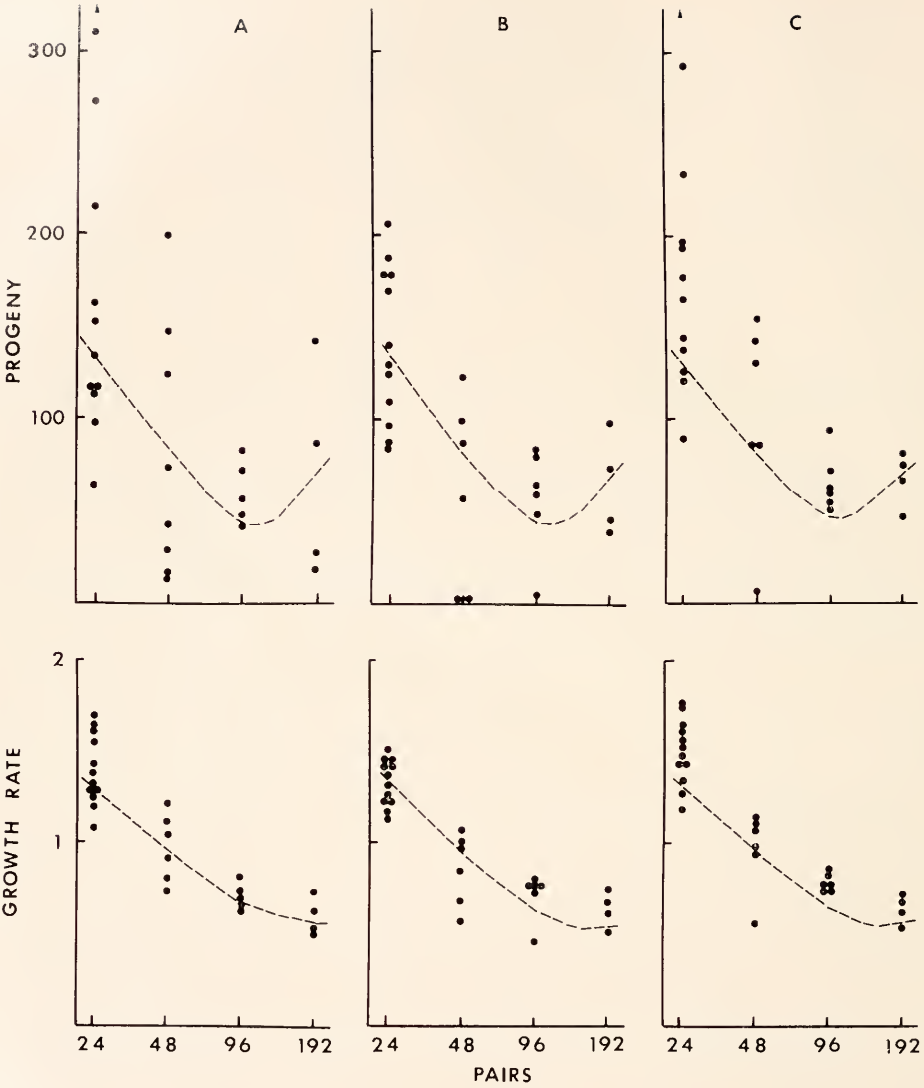

FIGURE 3. Total progeny (above) and finite population growth rate (below) as functions of the number of pairs of parental flies in the density experiment for three initial frequencies of *s* fourth chromosomes: A = 5/16, B = 8/16, C = 11/16. Dotted lines are the respective common (over initial fourth chromosome frequency) regressions for total progeny: $\ln(\text{progeny} + 1) = 5.5050 - 0.0139N(t) + 0.000028N^2(t), (r^2 = 0.179)$ or for finite population growth rate: $\ln(\lambda) = 0.6168 - 0.0073N(t) + 0.000012N^2(t), (r^2 = 0.788)$.

male progeny, while in the stocks experiment similar cultures produced a slight excess of male progeny. Ignoring these deviations, the sex ratio averaged 0.92 in the strains experiment, 1.07 in the stocks experiment, and 1.01 in the viability experiment; none of these values is statistically different from 1. Because estimates

TABLE II

Adjusted mean total progeny and finite population growth rate of karyotypes in monomorphic cultures.

Karyotypes	Experiment: Strains	Stocks	Viability
A. Total progeny			
s/s	291.4	197.9	129.6
c/c	191.1	157.9	108.7
s/c	267.4	271.8	—
c/s	252.5	258.2	—
Heterokaryotypic parents	—	—	77.9
{Slope}	$\{-0.008336N + 0.0000833N^2\}$	$\{-0.009806N\}$	$\{-0.002611N\}$
B. Finite population growth rate			
s/s	1.2685	1.3455	.9881
c/c	1.1070	1.2564	1.0771
s/c	1.2174	1.4528	—
c/s	1 2244	1.4568	—
Heterokaryotypic parents	—	—	.8934
{Slope}	$\{-0.004638N + 0.00004098N^2\}$	$\{-0.005532N\}$	$\{-0.002930N\}$

N = number of parents introduced into cultures.

were variable and inconsistent between series, there is no compelling reason to assume the sex ratio of progeny of any of the genotypes is not 1 for purposes of population prediction.

The variation between experiments makes these results difficult to interpret (Cohen, 1976). The overall impression is that the karyotypes do not differ greatly from one another in the absence of interkaryotypic competition.

Population dynamics under selection

Dynamics of populations polymorphic for fourth chromosomes were measured in two experiments. The first of these, hereafter called the *composition experiment,* was intended primarily to measure the influence of population composition. Experimental design is detailed in DeBenedictis (1978). Briefly, seven mixtures of *s/s, s/c, c/s* and *c/c* karyotypes (Table III), each totaling 120 flies, were established. Cultures were censused as in the stocks experiment, except that censuses were continued through day 24 owing to the low productivity of cultures on the census taken on day 12. The seven experimental treatments were little altered by mortality of parental flies, and all converged toward a composition of about 60% *s* fourth chromosomes (DeBenedictis, 1978).

Most cultures in this series exhibited negative net population growth (Table III; Fig. 5). There were no detectable differences between treatments in the number of offspring obtained on any of the census dates, but there may be a difference

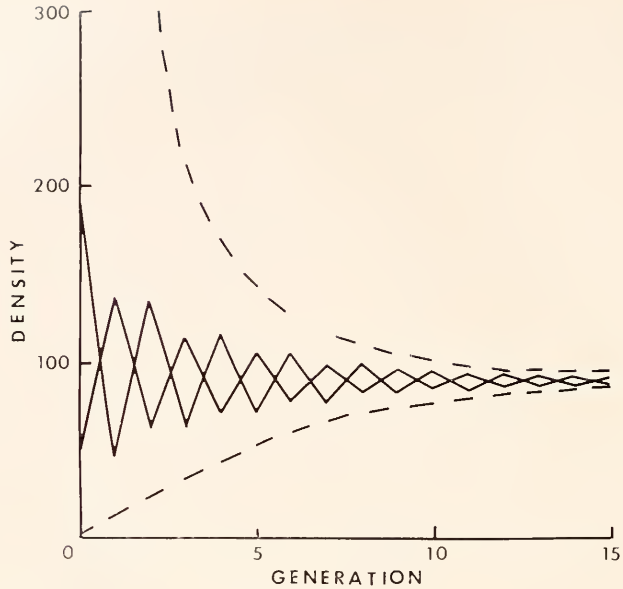

FIGURE 4. Representative discrete generation trajectories of populations obeying the rule: $N(t+1) = 245.9\ \exp(-0.0139N(t) + 0.000028^2(t)) - 1$ (*cf.*, Fig. 3). Solid lines give trajectories for populations with $N(0) = 48$ or 192. Dotted lines indicate the regions within which all trajectories with $2 \leq N(0) \leq 548$ must lie.

in mean total progeny obtained/culture. No difference was detectable when ln(total progeny) were subject to analysis of variance, but the largest treatment mean was statistically greater than the three smallest treatment means (the latter means differ only from the largest) when (total progeny)$^{\frac{1}{2}}$ were subject to the same analysis; the log transformation produced somewhat more homoskedostic treatment variance. However, the largest mean finite rate of increase (Table III) was significantly different ($F_{6,52} = 2.39$, $P = 0.04$) from the smallest, and *vice versa,* although no other means were statistically distinct *a posteriori.* There was no obvious relationship between the composition of parental populations and any of these parameters.

TABLE III

Influence of population composition.

Parental population composition			Mean (± s.e.) progeny obtained on					Mean (± s.e.)
s/s	*s/c*	*c/c*	Day 12	Day 16	Day 20	Day 24	Total	
2	88	30	0.25 ± 0.15	10.8 ± 4.7	14.0 ± 4.5	20.9 ± 2.6	52.0 ± 7.0	0.8336 ± 0.0253
30	60	30	0.67 ± 0.35	11.9 ± 2.5	17.7 ± 2.5	23.0 ± 2.5	55.5 ± 6.3	0.8436 ± 0.0183
30	88	2	0.12 ± 0.12	14.4 ± 3.2	18.8 ± 3.4	23.5 ± 2.3	60.3 ± 5.3	0.8648 ± 0.0161
10	100	10	0.22 ± 0.14	10.2 ± 3.5	20.6 ± 2.9	26.9 ± 3.8	62.9 ± 7.2	0.8691 ± 0.0200
50	20	50	0.44 ± 0.32	16.0 ± 7.5	21.6 ± 2.3	27.6 ± 5.0	72.1 ± 2.9	0.8946 ± 0.0316
30	20	70	0.50 ± 0.25	17.3 ± 5.2	25.8 ± 2.2	24.0 ± 5.9	72.3 ± 10.5	0.8972 ± 0.0254
70	20	30	0.62 ± 0.35	27.3 ± 4.7	23.3 ± 3.8	34.2 ± 4.5	89.6 ± 6.4	0.9441 ± 0.0120

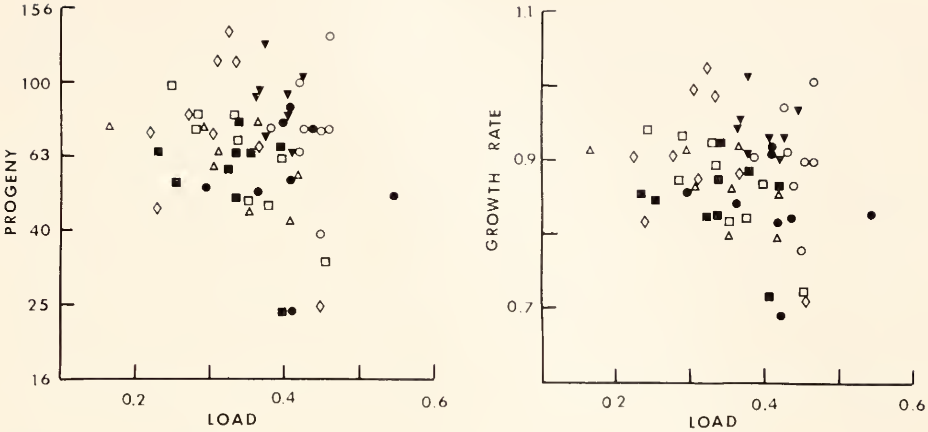

FIGURE 5. Total progeny (left) and finite population growth rate (right) versus theoretical genetic load for seven initial popoulation compositions indicated as in Table III: open circles = 30/20/70; solid circles = 2/88/30; open squares = 10/100/10; solid squares = 30/60/30; open diamonds = 50/20/50; open triangles = 70/20/30; and solid triangles = 30/88/2. Loads differ between initial population compositions but are uncorrelated with total progeny or λ.

In the second experiment, hereafter called the *density experiment*, both population composition and density were varied. Experimental design and gene dynamics are described elsewhere (DeBenedictis, 1977). Briefly, experimental design was factorial, with three levels of the factor, population composition: 3/16, 8/16, and 11/16 frequency of *s* fourth chromosomes in parental flies; and with four levels of the factor, population density: 24, 48, 96 and 192 pairs of parental flies (Fig. 3). For genetical reasons (DeBenedictis, 1977) two series of cultures containing 24 pairs of parents were required; these were treated as separate levels of the factor density in analyses of variance. Each treatment at the highest density level had four replicates, while each treatment at the lower density levels had six replicates. Results from one anomalous culture were omitted.

Two-way analysis of variance of ln(total progeny + 1) and of ln(λ) revealed only initial population density to have detectably influenced their value (Fig. 3; Table IV). Crowded populations generally grew less rapidly than uncrowded

TABLE IV

Analyses of variance of the density experiment.

Source	d.f.	Mean square		
		Total progeny	Finite rate of increase	Sex ratio
Initial density	4	5.1160*	2.0399*	0.0448*
Initial composition	2	1.6837	0.0440	0.0087
Interaction	8	0.6401	0.0056	0.0034
Within treatment	68	0.6478	0.0218	0.0077

* Associated F-ratio has probability < 0.01 if treatment means are equal.

TABLE V

Analyses of covariance of the density experiment.

Adjusted mean	Initial frequency of spa^{pol} + + fourth chromosomes			Mean square		$F_{2,78}$
	0.3125	0.5000	0.6875	Between treatment	Within treatment	
Total progeny	99.65	67.40	106.88	0.3117	0.1245	2.543
Finite rate of increase	0.9933	0.9516	1.0296	0.0082	0.0043	1.923

populations but logarithmic regressions of initial density on total progeny and on λ were significantly curvilinear (Fig. 3), as in the strains experiment. Similar results apply to the number of progeny obtained on each census of these cultures. Covariance analysis supports these conclusions (Table V); no differences in adjusted means are detectable. Identity of these regressions implies that carrying capacity also was independent of population composition. Although only three levels of initial population composition were compared in this experiment, the range of compositions was nearly as great as in the composition experiment, and further, sample size for each composition was larger. The only reasonable conclusion from these results is that population composition has little influence on the population dynamics for this genetic system, even though these populations experienced intense natural selection.

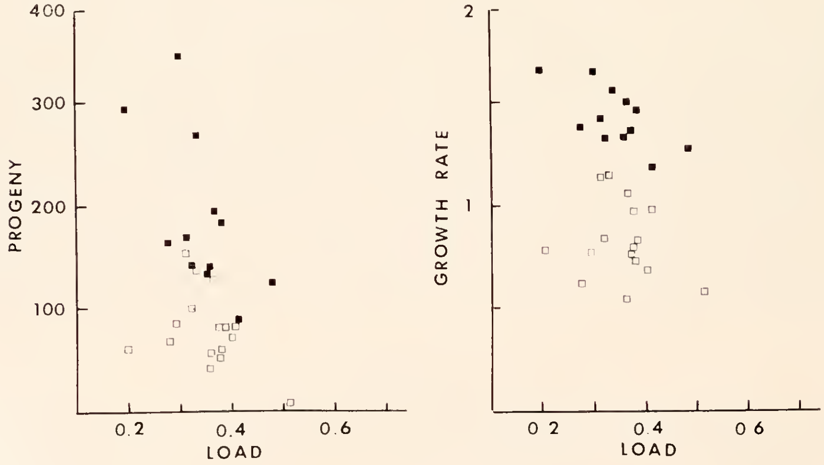

FIGURE 6. Total progeny (left) and finite population growth rate (right) *versus* theoretical genetic load in the density experiment for cultures initially with 11/16 *s* fourth chromosomes; solid squares are the lowest density level; open squares are the three higher density levels. If, as illustrated, the demographic variables are not adjusted to a common density, no relation exists; when adjusted to that expected from a common initial population density, a relation similar to that indicated by the solid squares exists. No correlation between load and demographic variables was evident in cultures that initially had 5/16 or 8/16 *s* fourth chromosomes in this experiment, even if demographic variables are adjusted for variation in initial population density.

The sex ratio was not influenced by initial population composition in the composition experiment ($F_{6,52} = 1.19$, $P = 0.33$), but its overall average of 1.21 was significantly > 1 ($t_{58} = 3.99$, $P < 0.001$). In the density experiment the sex ratio averaged 0.80, a value statistically < 1 ($t_{82} = -19.16$, $P < 0.001$), and was also influenced by initial population density (Table IV); sex ratios were highest but not distinguishable in cultures initiated with 24, 48 or 96 pairs of flies, but the sex ratio in cultures initiated with 192 pairs of flies was not distinguishable from that in cultures initiated with 48 pairs. Barker and Podger (1970a) also report skewed sex ratios, which were influenced by increasing population density, but their experimental design precludes more detailed comparison with the present results. Because sex ratios in these experiments deviated from unity in opposite directions, a constant sex ratio of 1 is assumed in the following discussion.

Discussion

Population dynamics in all experiments were most easily compared by adjusting mean parameter estimates to that corresponding to a constant initial density of 120 flies, the only density used in the composition experiment. This comparison revealed that population growth rates generally declined during the course of this study; they were lowest in the composition experiment. However, this difference cannot be attributed to the fact that in some experiments populations were monomorphic while in others they were polymorphic; population growth parameters in the density experiment (with polymorphic cultures) were indistinguishable from those in the viability experiment (with mostly monomorphic cultures). Barker and Podger (1972a, b) found that time influenced *Drosophila* population dynamics even within their experiments, and Wade (1977) provided data for a *Tribolium* system that exhibited a similar inexplicable decline.

Though variability of demographic parameters limits their utility in predicting growth, two features were consistent in all experiments. First, population growth declined with increasing population density. Secondly, because offspring percapita declined less rapidly than density increased, in very crowded cultures there was a slight increase in total offspring and in λ at the highest density levels, as in the strains (Fig. 2) and density experiments (Fig. 3). This second feature is also suggested in the other experiments where use of parabolic regression could not be statistically justified. Use of parabolic regressions for population prediction has two unrealistic features: they permit population growth when population density is 0 (or negative), and population size can expand indefinitely should it become sufficiently large. Fortunately, the empirical curves imply sufficiently strong population regulation that neither of these events can occur unless they are adopted as initial conditions. Further, it is likely that, were density increased beyond the levels tested in the present study, reproductive output would again decline with increasing crowding in this system.

Populations growing according to the empirical equations (Fig. 3) will approach a stable equilibrium density with oscillatory damping (May and Oster, 1976) whenever populations are initially small enough that "exponential" growth is not possible. If it were possible to reproduce consistently the environmental conditions under which these demographic parameters were measured, and if flies do not evolve with respect to these conditions, discrete generation population growth may be predicted from the relations indicated in Figure 3; or from the equation: $\ln(\text{progeny} + 1) = 5.0725 - 0.0179N + 0.00003159N^2$ ($r^2 = 0.306$), which relates the number of offspring obtained between days 12 and 16 in the density experiment to parental density, N. This last equation represents discrete generation population dynamics better than does the equation for total progeny, since too few offspring matured before day 12 in high density cultures to permit use of the corresponding regression for population prediction. Depending on which equation is used, a population will converge, slowest for the above equation, to an equilibrium density of approximately 90 flies. Representative trajectories, and the limits within which all trajectories must lie provided $2 \leq N_0 \leq 548$, are shown in Figure 4. These simulations ignore the great stochastic variation in reproduction output; Shorrocks (1970) ably discussed the influence of such variation.

Gene dynamics were utterly distinct. Adaptive values estimated from the composition experiment were consistent with those from the density experiment. Further, adaptive values proved quite sensitive to population composition but were uninfluenced by variation in population crowding (DeBenedictis, 1977, 1978). Simulation of gene dynamics predicted that genetic equilibrium effectively would be reached in a maximum of five generations over the range of population compositions tested in these experiments (DeBenedictis, 1977). These differences in response and in time scale indicate that gene and population dynamics were independent in this system, and are particularly interesting relative to genetic load theory (Crow, 1970; Crow and Kimura, 1970; Lewontin, 1974; Wallace, 1970).

Loads are usually measured by comparing inbred with randomly mated strains. The present study makes possible measurement of the effects of a "segregation" or

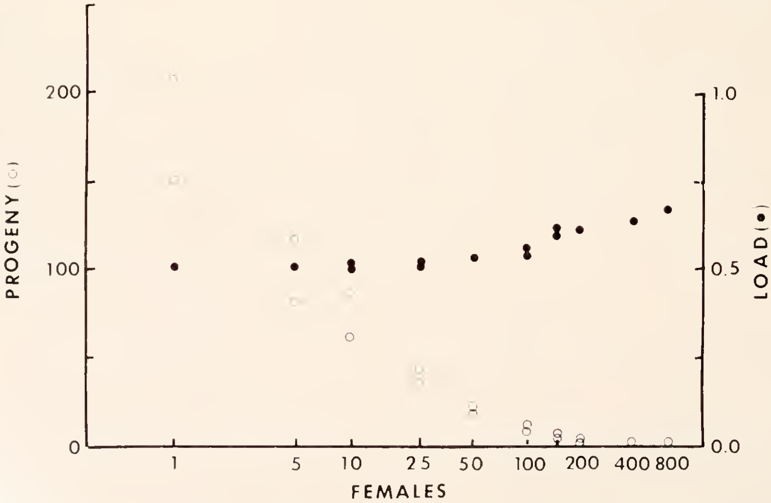

FIGURE 7. Mean total progeny/female parent (open circles) and mean segregational load (solid circles) as functions of initial female parental density for the *ebony* locus in *Drosophila melanogaster*. Values recalculated from Moree and King (1961: Table 3); dots indicating load for 1, 5, and 50 females represent two identical estimates.

"heterotic" (Wright, 1977) load for these *Drosophila* populations. The load, $L = 1\text{-}w$ (Crow, 1970), was calculated from the adaptive values estimated in DeBenedictis (1977, 1978). The present report provides demographic characteristics for the same populations. If the load has an ecological impact, then total progeny and/or the finite growth rate should decline as the load increases. In the composition experiment loads calculated for the different initial population compositions (Fig. 5) were heterogeneous ($F_{6,52} = 15.00$, $P < 0.001$). Within treatments the demographic parameters tended to decline as the calculated load increased, but only one correlation was statistically significant (Fig. 5); between treatments the demographic parameters tended to increase as the load increased, but the correlation was indistinguishable from 0. In the density experiment calculated loads were insensitive to initial population density ($F_{4,68} = 1.35$) and there was no factor interaction ($F_{8,68} = 2.05$); however, loads differed significantly between levels of initial population composition ($F_{2,68} = 10.41$, $P < 0.01$). Expression of the load could be confounded by the dependence of population growth parameters upon population crowding (Fig. 6), unless these parameters are adjusted by covariance analysis to the values expected if all cultures had begun at a constant initial density. Such analysis revealed the expected relationship between load and dynamics only in cultures whose composition initially was 11/16 *s* fourth chromosomes (as in solid squares, Fig. 6); no correlation was evident between treatments. There has been no detectable influence of the "segregational load", other than changes in the genetic composition of these populations, even though the load may differ nearly two-fold between treatments.

While these results are anticipated by some theories, experimental substantiation is sparse because so few genetical studies have measured possible influences of crowding. Most of studies of density-dependent selection cited in the introduction to this report describe only larval viability. An investigation of the *ebony* locus of *Drosophila melanogaster* by Moree and King (1961) is the most nearly comparable study. Adaptive values, calculated as in DeBenedictis (1977, 1978), for their data were: $w_{+/+} = 0.92$; $w_{+/e} = 1$; and $w_{e/e}$ declined with population density. Because all parental flies in their study were heterozygotes, the load for these populations was $L = 0.73 - 0.25w_{e/e}$ and increased with density. Population growth declined with increasing density, but because only one genetic composition was described, it is unclear to what extent this decline owed to ecological limits or to the increasing segregational load. If the load were the main determinant, then population growth, measured as mean progeny/parent, should decline in parallel with increases in selectional load. It did not (Fig. 7).

These examples could be criticized because they involve genes that disrupt the phenotype to an exceptional degree relative to most natural genetic variants, and because laboratory conditions shelter the organisms from natural environmental heterogeneity. Such criticism would be more important had a relationship between load and population dynamics consistently been evident, because the reduced effects of natural genetic variants and heterogeneity of natural environments may be invoked to explain how free-living populations tolerate their genetic loads. If one anticipates such mitigating effects, as most theory (*e.g.*, Levins, 1968) does, then the present experiments enhance the probability of observing any detrimental effects of a segregational load. If *D. melanogaster* can tolerate genes such as these under "constant" laboratory conditions, then surely it can tolerate most natural genetic variants under natural conditions.

Genetic loads that accompany inbreeding depression often can be associated with fixation of genes whose deleterious effects are evident even in monomorphic cultures. The present study is quite different, because all three karyotypes involved theoretically can sustain vigorous monomorphic populations; indeed, the only difficulty is the biological impossibility of keeping heterokaryotype cultures monomorphic. Yet homokaryotypes are greatly disadvantaged in polymorphic cultures, and these *Drosophila* populations are responsive to population crowding. Why is there no load, and why are gene and population dynamics independent?

Frequency-dependent selection, although pertinent (DeBenedictis, 1977) and sometimes invoked to explain away loads, cannot be the explanation in this system. Not only was no load evident under nonequilibrium conditions, but a strong load is expected even under genetic equilibrium (DeBenedictis, 1977), as in several other genetic systems that exhibit frequency-dependent selection (DeBenedictis, 1978). Rather, the explanation seems to derive from the mechanism that produces frequency-dependent selection and from similar response of each karyotype to population crowding. Frequency-dependent selection appears to be generated almost entirely by female mating preferences: females reproduce essentially in proportion to their relative frequency and egg production is independent of the genotype of their mate, while the success of a male is strongly biased by its genotype (Bundgaard and Christiansen, 1972). Because biotic potential of the parents is not altered by this pattern of matings, differential mating success permits extensive genetic change without deleterious demographic consequences. Further, characterization of the karyotypes in isolation revealed that while their absolute (Darwinian) fitness declined with increasing crowding, the decline was parallel for all karyotypes. Therefore, their relative fitness, which determines the rate of genetic change, remained constant. Exactly the same behavior is evident in some other studies of "density-dependent selection" (notably Sokal and Huber, 1963; and Sokal and Karten, 1964), for which the published data also suggest no influence of crowding on the rate of gene change (DeBenedictis, 1977).

The theoretical explanation that seems to best apply to this system is "soft selection" (Wallace, 1968a, b, 1970, 1975). While these data appear to provide the clearest experimental validation of this concept, they must qualify Wallace's otherwise lucid expositions. The first point concerns the distinction between Darwinian fitness (mean offspring/parent) and relative fitness. Wallace correctly notes the ecological difference: Darwinian fitness of a population must equal or exceed one for a population to persist in time. However, his discussions do not sufficiently emphasize that it is the ratio of Darwinian (*i.e.*, relative) fitnesses that determines gene dynamics. Thus, given a set of relative fitness values, evolution occurs equally rapidly whether population size is expanding, declining, or constant. To that extent, the load is a mathematical artifact no matter how fitness is measured.

Wallace's emphasis on Darwinian fitness leads him (1975) to restrict "soft selection" to those cases in which selection is both frequency-dependent and density-dependent; and to restrict its opposite, "hard selection", to frequency- and density-independent selection. While the latter is necessarily true, "soft selection" should be more broadly applied to include all cases in which the only demographic responses to natural selection are changes in the genetic composition of the population This modified definition of soft selection with respect to frequency or density-dependent selection only blurs the distinction Wallace (1975) has made without challenging the essence of his discussion. By my criterion, the system described in this report is an example of frequency-dependent, density-independent selection which also exhibits "soft selection". Since it is conceivable that frequency-dependent, density-independent selection sometimes could influence the demography of populations, and since this is likely to be the case under density-dependent, frequency-independent selection, it will be necessary to examine a broader array of genetic systems to determine how widespread "soft selection" is.

Helpful reviews of earlier versions of this report by D. L. Sullivan and two anonymous reviewers are most gratefully acknowledged. I remain responsible for any errors and ambiguities that remain in spite of their comments. M. Cote provided invaluable assistance in the laboratory, and D. L. Lundgren generously provided funding from the Department of Biology, Syracuse University.

SUMMARY

1. Growth of *Drosophila melanogaster* populations which were simultaneously subject to intense natural selection for marked fourth chromosomes was characterized in terms of number of progeny and a finite population growth rate.

2. There is no indication that the conditions experienced by past generations influenced the population growth potential of a cohort of adult flies; that is, population dynamics possess Markovian properties, justifying their characterization from observations of single-generation transitions.

3. The three karyotypes studied in this system exhibited parallel reductions in growth rates to increasing population density; they differed in "carrying capacity" or equilibrium density inconsistently and to a lesser degree than was suggested by adaptive values.

4. Experimental variation of the genetic composition of parental cohorts had almost no effect on population dynamics, but population growth rates declined markedly with increasing population density. Selection in this system has been found to be density-independent, but fitness of each genotype depends on population composition.

5. As a consequence of point 4, there is no discernable relationship between gene and population dynamics in this system. In particular, the theoretical "segregational load" and population growth rates were statistically uncorrelated.

6. This system is discussed in relation to the concept of soft selection. These data suggest that the definition of soft selection is less precisely related to the concepts of density-dependent and frequency-dependent selection than has been proposed.

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THE EFFECT OF AGE AND DIAPAUSE ON THE LONG-TERM INTAKE OF PROTEIN AND SUGAR BY TWO SPECIES OF BLOWFLIES, *PHORMIA REGINA* (MEIG.) AND *PROTOPHORMIA TERRAENOVAE* (R. D.)

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Insects have proved to be good model systems with which to study feeding behavior. Some insects, in particular the black blowfly, *Phormia regina,* are capable of regulating their food intake. This species has been the subject of feeding studies in which both the maintenance of metabolic homeostasis and the underlying regulatory mechanisms have been investigated.

The principal food of the blowfly is carbohydrate (Dethier, 1969), although Strangways-Dixon (1961), Dethier (1961) and Belzer (1970) have demonstrated the existence of a specific protein hunger in flies. Female blowflies increase selectively their consumption of proteins prior to periods of egg maturation resulting in the appearance of cycles of protein intake concommitant with reproductive cycles. Spermatogenesis occurs independent of feeding in the blowfly (Cowan, 1932; Mackerras, 1933; Stoffolano, 1974a), although male specimens of *P. regina* require a protein meal for the maturation of the accessory reproductive glands (Stoffolano, 1974a). Male blowflies exhibit an initial peak of protein consumption but do not show subsequent peaks of protein intake (Belzer, 1970; Roberts and Kitching, 1974).

Studies on aging in insects (Clark and Rockstein, 1964; Rockstein and Miquel, 1973; Stoffolano, 1976) have indicated that numerous degenerative structural and functional changes occur in aging adult insects, such as mechanical damage to cuticle and wings, reduced fecundity, reduced levels of protein synthesis and respiration rates, and degeneration of the central nervous system. Changes occurring in the structure and function of the nervous system, specifically the peripheral chemoreceptor sense organs, are of particular interest since these receptors are intimately involved in the feeding process. A receptor system which showed age-related degenerative changes would imply that aged flies are less efficient feeders. Aged flies show increasing numbers of nonfunctional labellar sensilla and among those that remain functional, a decrease in the mean impulse frequency occurs (Rees, 1970; Stoffolano, 1973; Stoffolano, Damon and Desch, in preparation).

Stoffolano (1973) reported the occurrence of a facultative imaginal diapause in *P. regina* characterized by the lack of ovarian development in the female and by hypertrophy of the fat body in both sexes. In addition, the accessory reproductive glands are undeveloped in the diapausing fly. The occurrence of an imaginal diapause has also been reported for *Protophormia terraenovae* (Roubaud, 1927; Cousin, 1932; Danilevskii, 1965). Behavioral alterations such as migration, reduced mating activity, and changes in feeding behavior may also occur during insect diapause. Prior to entry into diapause many insects feed ravenously to build up large reserves of fat and glycogen in the fat body and other storage tissues (Lees, 1955). However, no work has been done on the selective intake of protein and sugar by diapausing flies, as compared to their nondiapausing counterparts (Stoffolano, 1974b).

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This paper presents the results of an investigation aimed at elucidating the effects of age and diapause on the long-term intake of protein and sugar by male and female *P. regina* and *P. terraenovae* maintained under controlled environmental conditions.

MATERIALS AND METHODS

Adult specimens of *Phormia regina* and *Protophormia terraenovae* were collected in 1972: *P. regina* in Ithaca, New York, and *P. terraenovae* in College, Alaska. Rearing procedures for stock colonies of both species were identical, utilizing the techniques and diet described earlier (Stoffolano, 1973).

Rearing of experimental colonies

Experimental colonies of nondiapausing flies were obtained from larvae reared at 24° C, 24 hr light and 50–60% relative humidity. Pupae were transferred to an environmental chamber where subsequent emerging adults were reared. Nondiapausing *P. regina* adults were reared under June conditions of daylength and temperature for the Ithaca, New York, area in an attempt to simulate those conditions experienced by the insects in nature. Adults received a 16 hr photophase with a temperature of 24° C during the light phase and 13° C during the dark phase (8 hr). Diapausing *P. regina* adults were obtained from flies reared under conditions described earlier by Stoffolano (1973). Larvae were reared at 24° C and 24 hr dark. Pupae were transferred to an environmental chamber where subsequent emerging adults received a 9 hr photophase and a temperature of 18° C.

A concommitant low temperature and short photophase is required by *P. regina* adults for induction of the diapause condition, whereas in *P. terraenovae,* diapause appears to be temperature-independent (Greenberg, personal observation); diapausing flies can be reared at the same temperature as the nondiapausing population, thus eliminating a possible differential temperature effect which might confound the results. Therefore, *P. terraenovae* was used to repeat those studies made with *P. regina* on the intake of protein and sugar by diapausing and nondiapausing flies.

Nondiapausing adult specimens of *P. terraenovae* were obtained from larvae reared similarly to those of nondiapausing *P. regina*. Pupae were transferred to an enviromental chamber where pupae and emerging adults received 24 hr light and 24° C. Diapausing flies were obtained from larvae reared in a manner similar to those of diapausing *P. regina*. Pupae were transferred to an environmental chamber, where emerging adults were kept at 24° C and a 9 hr photophase.

Large numbers of larvae were reared in order to obtain enough flies of approximately the same age. Only those flies emerging during one twelve-hour period were used; all others were discarded. The maximum age difference between any two flies was thus twelve hours.

Adult flies were maintained in approximately equal numbers in 25 × 50 cm wire mesh cages with front sleeving. Paper toweling, which was charged weekly, was placed on the bottom of the cages to absorb liquid wastes. The cages contained standard feeding solutions offered in separate glass vials stoppered with absorbant rolled cotton wicks. Feeding solutions consisted of 10% (w/v) yeast extract (Nutritional Biochemicals, Co) and 0.1 M sucrose prepared in distilled water. These solutions, offered as standard diet to the experimental colonies, were identical to the solutions used in the two-choice testing experiments. The feeding solutions were prepared fresh daily. Water was also available to the experimental colonies and was changed weekly. The artificial diet (Stoffolano, 1973) on which the larvae were reared was used as an oviposition medium in a manner similar to that described by Orr (1964). This eliminated an additional source of protein in the experimental colonies, since flies were observed to oviposit but not to feed on the medium. Flies were also observed to oviposit readily on the rolled cotton wicks saturated with feeding solutions.

Cages were housed in an environmental chamber equipped with four 20 watt fluorescent lights. Flies received a photophase and temperature regime as described earlier. Average light intensity in the chamber was 3121.63 lux. Humidity in the chamber fluctuated between 50–60% relative humidity.

Measurement of intake

Food intake was measured using a two-choice apparatus, consisting of individual feeding units housed in a clear plexiglass container, adopted and modified from Belzer (1970).

Individual feeding units consisted of two precision bore glass capillary tubes (internal diameter 0.889 mm, 30.5 cm long) inserted through holes drilled in a rubber stopper which in turn fitted into a cellulose nitrate centrifuge tube, 3 cm in diameter and cut to 7.5 cm in length with nylon mesh glued to the cut end. The glass tubes, calibrated externally in mm, were obtained from Ace Glass, Co., Vineland, New Jersey. The use of these tubes, rather than those used by Belzer (1970), provided more accurate measurements of intake. A further benefit of the calibrated capillary tubes was that it was no longer necessary to open the plexiglass container in which the tubes were housed to take readings. Readings could be taken through the plexiglass container, thus subjecting humidity inside the container to fewer fluctuations.

The clear plexiglass container measured 25 × 36 × 38 cm. The individual feeding units housed in the container were secured with rubber tubing stretched on removable plexiglass platforms. The platforms rested on adjustable wooden racks which could be tilted to produce an incline such that the fluid in the capillary tubes would flow downward as the fly fed. Relative humidity in the plexiglass container was maintained at 60–70% with a saturated NaCl solution prepared according to Winston and Bates (1960) and placed in a pan on the bottom of the container. The entire apparatus was housed in an environmental chamber equipped with four 20 watt fluorescent lights. Relative humidity in the chamber was maintained at 50–60%.

The standard feeding solutions were introduced into the glass capillary tubes using a hypodermic syringe and were then allowed to equilibrate for approximately

2 hr during which time evaporation among the capillary tubes was found to be the greatest. One tube of each feeding unit contained the 10% yeast extract solution ("protein" solution); the other contained 0.1 M sucrose or sugar solution. A "zero" reading was taken at the end of this period, and the cellulose nitrate centrifuge tubes containing the flies were fitted into place. Final readings were taken the next day (22 hr later); all readings were taken at the same time each day. At the end of each feeding trial capillary tubes were removed and replaced with clean ones, and the flies were discarded or dissected (see below). Used tubes were washed in detergent solution, rinsed in tap water and dried in an oven at 75° C.

Seven flies of each sex were used each day in calculating the daily mean intake. New flies were taken each day from the experimental colonies and briefly anesthetized with CO_2. The flies were separated as to sex, weighed individually and transferred to the cellulose nitrate centrifuge tubes, one fly to a tube. An equal number, seven, of evaporation controls (consisting of the individual feeding units without flies) were run concurrently. Evaporation among the capillary tubes was found to be least in those tubes closest to the salt bath and greatest among those farthest away. Controls and flies were therefore distributed as to their position on the plexiglass platforms using a table of random numbers to minimize variations in the results due to differential evaporation. Intake was measured from day 1 to day 40 for *P. regina* and from day 1 to day 25 for *P. terraenovae*. Flies were tested at the same temperature and photophase regime under which they were reared, except diapausing specimens of *P. regina* which were tested at the same temperature and photophase regime as the nondiapausing population.

Dissections

Female flies were dissected in an insect saline solution (Normann and Duve, 1969) at the termination of each day's feeding trial, and ovarian measurements taken to determine the relative stage of development. Diapausing flies were also dissected and examined for fat body hypertrophy and ovarian development.

Statistical analysis

Analysis of variance of the data was used to determine the effect of sex, age, diet and treatment (diapausing or nondiapausing) and their interactions on the intake of the fly. This included computer calculation of means, standard error of the mean, and F-values. An analysis of variance was also made of evaporation data, and these results were used in calculating adjusted daily mean intake values. Each point in Figures 1–4 represents the daily mean intake (in μl) for seven flies minus the mean evaporation for that day, for that diet and for that treatment.

An analysis of covariance was carried out to determine the effect of fly weight on intake.

RESULTS

Nondiapausing and diapausing P. regina

The intake of protein and sugar by nondiapausing male and female *P. regina* was measured from day 1 to 40 days after emergence. The results are shown in Figure 1 and Table I.

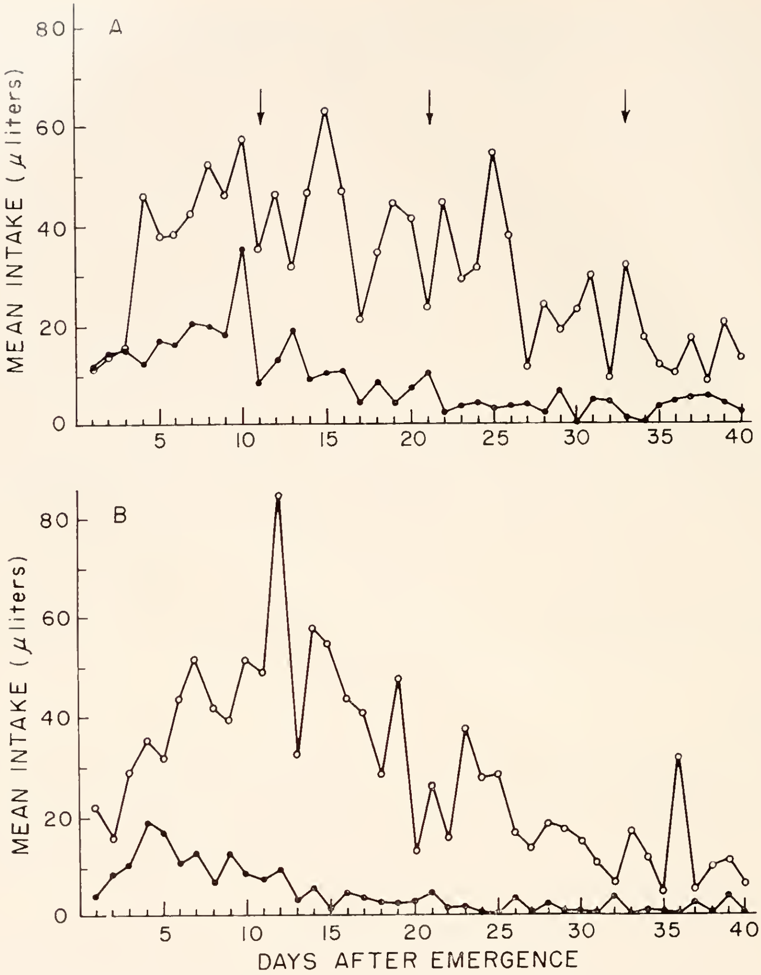

FIGURE 1. Total daily mean intake (μl) of protein (solid circles) and sugar (open circles) by nondiapausing female (A) and male (B) *P. regina* from day 1 to 40 days after emergence. Each point on the graphs represents the daily mean intake for seven flies minus the mean evaporation for that day and for that diet. Arrows indicate days on which eggs were laid.

Analysis of variance showed the total daily mean intake of protein and sugar by the population of flies was significantly influenced ($P < 0.01$) by the age of the fly. Intake was low during the first three days, increased on day 4 through 16, and then gradually decreased until day 40. Both sexes showed a similar trend in food intake with respect to age.

The total daily mean intake of sugar by the population of flies was significantly greater than protein intake. Females had a greater ($P < 0.01$) total daily mean intake compared to males and consumed both more sugar and protein than males. On the average, female flies weighed more than male flies (see Table I).

Analysis of covariance of the effect of fly weight on intake showed that when intake was considered on a μl/mg per day basis, sugar intake and total intake of protein plus sugar was not significantly different between male and female flies.

The intake of protein and sugar by diapausing male and female *P. regina* was measured from day 1 to 40 days after emergence. Diapausing flies were identified by lack of ovarian development in females and fat body hypertrophy in both sexes; the latter was not recognizable until day 15 after emergence. The results are shown in Figure 2 and Table I.

Analysis of variance showed age had a significant effect ($P < 0.01$) on the total daily mean intake of protein and sugar by the population of flies. Intake was low following emergence, increased rapidly on days 2 to 3, stayed high until days 27 to 28, then declined until day 40. Food intake by females was significantly more influenced ($P < 0.01$) by the age of the fly.

The total daily mean intake of sugar by the diapausing flies was significantly greater ($P < 0.01$) than protein. Females had a significantly greater ($P < 0.01$) total daily mean intake than males and consumed more of both protein and sugar compared to males.

On the average, diapausing female flies weighed more than diapausing males (see Table I). However, analysis of covariance of the effect of fly weight on intake (μl/mg per day) gave the same results as the μl/day analysis.

An analysis of variance conducted on data from nondiapausing versus diapausing *P. regina* showed that diapausing flies fed significantly more ($P < 0.01$) than nondiapausing flies. Intake of protein by the two populations of flies was not

TABLE I

Daily mean intake of protein and sugar by nondiapausing and diapausing Phormia regina *and* Protophormia terraenovae.

Experiment	Sex	Mean weight (mg)	Daily mean intake					
			Protein		Sugar		Protein and Sugar	
			(μl)	(μl/mg)	(μl)	(μl/mg)	(μl)	(μl/mg)
ND, *P. regina*	M	43.67	4.35	0.0996	28.24	0.6466	16.29	0.3730
	F	50.97	8.98	0.1761	30.40	0.5964	19.68	0.3861
	M + F	47.32	6.66	0.1407	29.32	0.6196	17.99	0.3801
D, *P. regina*	M	40.38	2.59	0.0641	46.34	1.1475	24.47	0.6059
	F	42.59	8.07	0.1894	51.97	1.2202	30.02	0.7048
	M + F	41.48	5.33	0.1284	49.16	1.1851	27.18	0.6552
ND, *P. terraenovae*	M	42.63	3.65	0.0856	45.23	1.0609	24.45	0.5735
	F	50.63	7.42	0.1465	47.51	0.9383	27.47	0.5425
	M + F	46.63	5.65	0.1211	46.26	0.9920	25.96	0.5567
D, *P. terraenovae*	M	46.67	4.36	0.0934	39.26	0.8412	21.80	0.4671
	F	49.86	6.04	0.1211	36.96	0.7412	21.50	0.4312
	M + F	48.26	5.20	0.1077	38.11	0.7896	21.65	0.4486

ND, nondiapause; D, diapause; M, males; F, females.

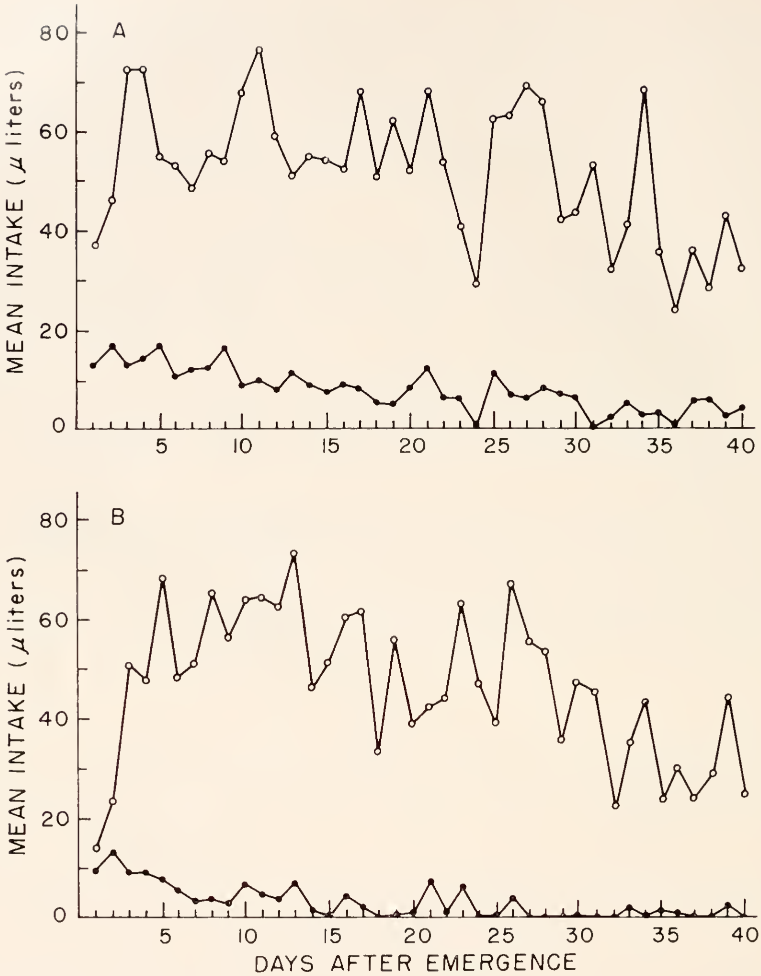

FIGURE 2. Total daily mean intake (μl) of protein (solid circles) and sugar (open circles) by diapausing female (A) and male (B) *P. regina* from day 1 to 40 days after emergence. Each point on the graphs represents the daily mean intake for seven flies minus the mean evaporation for that day and for that diet.

significantly different; but diapausing flies fed significantly more ($P < 0.01$) on sugar than the nondiapausing flies. In both experimental situations, females had a greater total food intake than males. There was a significantly greater ($P < 0.01$) effect of age on the total daily mean intake of the diapausing population compared to the nondiapausing population.

Diapausing individuals of *P. regina* weighed less, on the average, than the nondiapausing populations. An analysis of covariance of the effect of fly weight on intake (μl/mg per day) gave results similar to those for the μl/day analysis.

Nondiapausing and diapausing P. terraenovae

The previous experiments were repeated with modifications using the arctic blowfly, *P. terraenovae*. The results on the intake of protein and sugar by nondiapausing *P. terraenovae* from day 1 to 25 days after emergence are shown in Figure 3 and Table I.

Analysis of variance showed age had a significant effect ($P < 0.01$) on the total daily mean intake of protein and sugar by the population of flies. Intake was low during the first day after emergence, increased on days 2 to 3, remained high

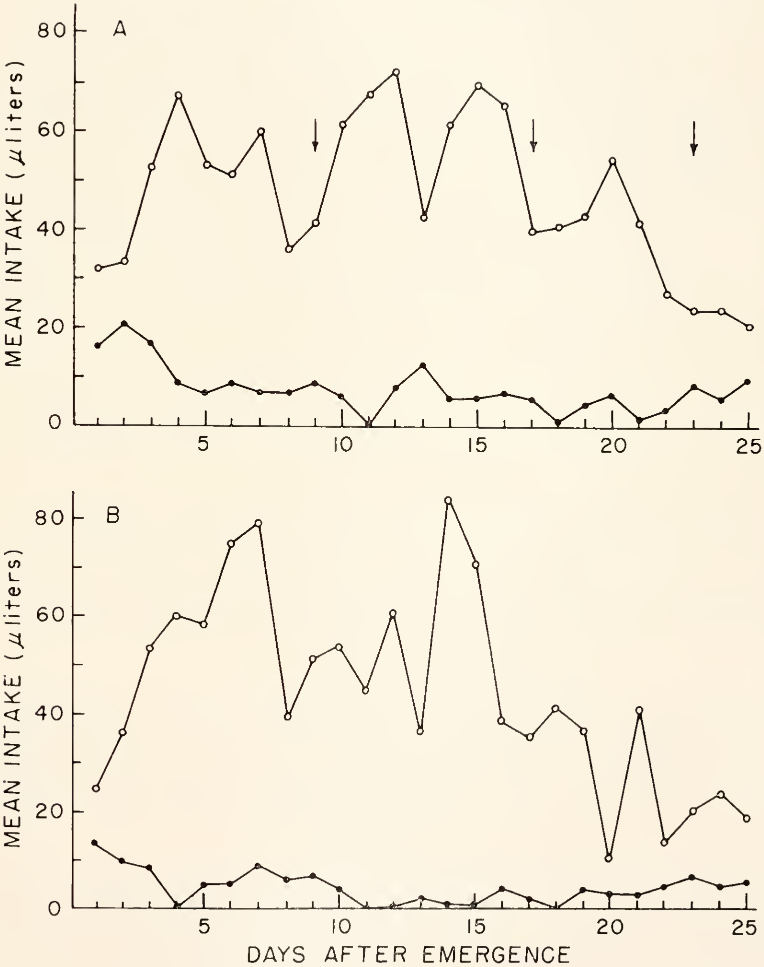

FIGURE 3. Total daily mean intake (μl) of protein (solid circles) and sugar (open circles) by nondiapausing female (A) and male (B) *P. terraenovae* from day 1 to 25 days after emergence. Each point on the graphs represents the daily mean intake for seven flies minus the mean evaporation for that day and for that diet. Arrows indicate days on which eggs were laid.

until days 14 to 15 and then declined steadily to day 25. Both sexes showed a similar trend in intake with respect to age. The total daily mean intake of sugar by the population of flies was significantly greater ($P < 0.01$) than protein. Females had a significantly greater total daily mean intake than males and consumed more of both protein and sugar compared to males.

In general, the results of analysis of covariance were similar to those for the nondiapausing *P. regina* population. That is, when intake was considered on a μl/mg per day basis, sugar intake and total intake of protein plus sugar was not significantly different between male and female flies.

The intake of protein and sugar by diapausing male and female *P. terraenovae* was measured from day 1 to 25 days after emergence. Diapausing flies were characterized by the absence of ovarian development in females and by fat body hypertrophy in both sexes. The latter was not apparent until 13 to 14 days after eclosion. The crop was observed to be full of a clear viscous fluid beginning about days 13 to 14. This was not seen in the diapausing *P. regina* population. The results are shown in Figure 4 and Table I.

Total daily mean intake of protein and sugar was significantly influenced ($P < 0.01$) by the age of the fly. Intake was low on day 1 after emergence, increased rapidly on day 2 to a high level which was maintained until day 12, when intake decreased rapidly until the last day of the experiment. This rapid decline in intake was not seen in the diapausing *P. regina* study. Both sexes showed similar trends in intake with respect to age.

Total daily mean intake of sugar by the population of flies was significantly greater ($P < 0.01$) than protein. No significant difference was found in the total daily mean intake of protein and sugar by diapausing females compared to males. These results are in contrast to those of diapausing *P. regina,* where diapausing females fed more than males.

Though diapausing female specimens of *P. terraenovae* consumed significantly more protein than did diapausing male *P. terraenovae*, the reverse was true for sugar intake; that is, diapausing males ate significantly more sugar than the diapausing females. These results are also in contrast to the diapausing *P. regina* population, where diapausing females fed more than males on both protein and sugar.

Diapausing female specimens of *P. terraenovae* weighed more than diapausing males. An analysis of covariance of the effect of fly weight on intake showed that when intake was considered on a μl/mg per day basis, the results were similar to those above, with the exception that sugar intake was not significantly different between males and females.

An analysis of variance was conducted on data from the nondiapausing and diapausing *P. terraenovae* populations to compare their intake. This analysis showed that the nondiapausing flies fed significantly more ($P < 0.01$) than did the diapausing flies. Protein intake by the two populations of flies was not significantly different, but nondiapausing flies consumed significantly more ($P < 0.01$) sugar than the diapausing flies. These results are different from those obtained with *P. regina,* where the diapausing population fed more than the nondiapausing population.

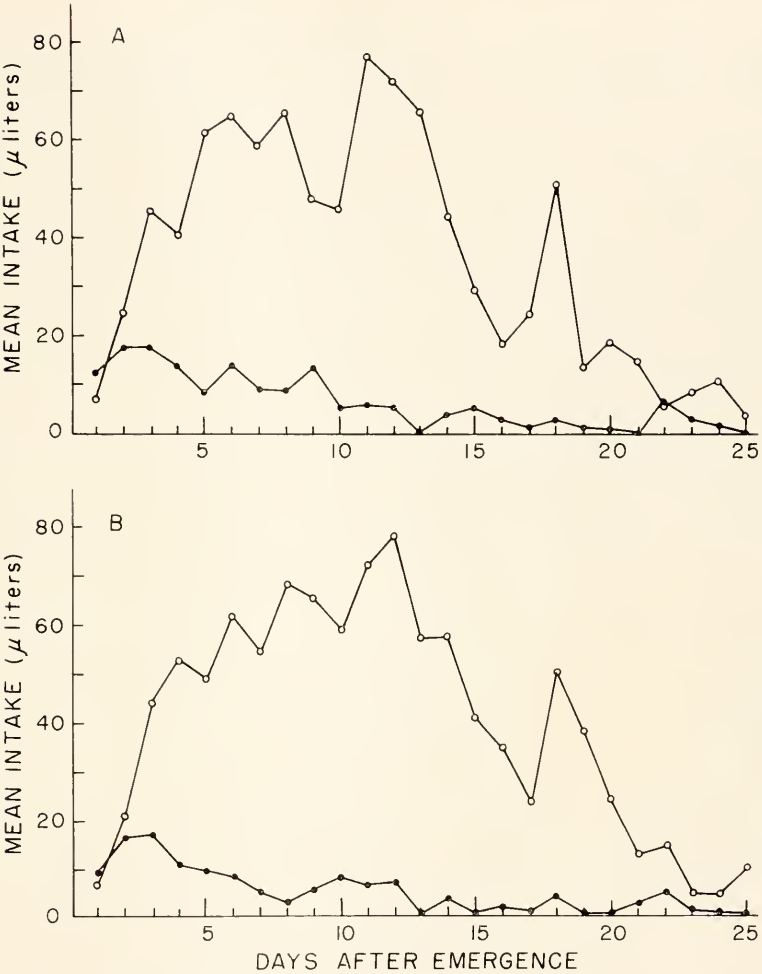

FIGURE 4. Total daily mean intake (μl) of protein (solid circles) and sugar (open circles) by diapausing female (A) and male (B) *P. terraenovae* from day 1 to 25 days after emergence. Each point on the graphs represents the daily mean intake for seven flies minus the mean evaporation for that day and for that diet.

There was a significantly greater ($P < 0.01$) effect of age on the total daily mean intake of the diapausing flies compared to the nondiapausing flies.

Diapausing flies weighed more than nondiapausing flies, on the average. An analysis of covariance of the effect of fly weight on intake showed that when intake was considered on a μl/mg per day basis, total intake of protein plus sugar was not significantly different between diapausing and nondiapausing flies. This is in contrast to what was seen for *P. regina,* where nondiapausing flies fed significantly less than diapausing flies on a μl/mg per day basis.

Discussion

Measuring the intake of protein and sugar by aging blowflies shows that there is a decline in intake with increasing age of the fly. This finding is consistent with other studies on food intake of aging flies (Dethier, 1961; Gelperin and Dethier, 1967; Roberts and Kitching, 1974). Greenberg (1959) found no age effect on the intake of protein and sugar by male and female houseflies between day 6 and day 20 after emergence. Among other Diptera, the intake of sucrose by aging female mosquitoes, *Aedes taeniorhynchus,* is generally similar to that observed for the blowfly (Nayar and Sauerman, 1974). In the present study, the effect of age on food intakes was similar for both sexes. This was also noted by Dethier (1961) for male and female blowflies feeding on sugar.

The causes underlying the general decline in food intake with age are not known. A review of the literature on aging in insects (*cf.,* Clark and Rockstein, 1964; Rockstein and Miquel, 1973; Stoffolano, 1976) reveals that numerous degenerative structural and functional changes occur in adult insects with age. Gelperin and Dethier (1967) suggested that the decline in food intake by aging flies could be due to several factors including decreased activity, accumulation of stored nutrient reserves or other subtle changes associated with senescence. The fact that aged flies are generally less active than younger ones may result in reduced energy or carbohydrate requirements for aged flies. The decreased respiratory rate with increasing age also suggests that aging flies probably need less food as they get older (Calabrese and Stoffolano, 1974). A decline in fecundity and in the number of eggs laid has been shown to occur among aged female insects (Greenberg, 1955; Woke, Ally and Rosenberger, 1956; Callahan, 1962) implying concommitant reduced nitrogen requirements. This might account for the decline in protein intake by aged female flies; however, no such clear-cut correlation can be found to account for the decline in protein intake by aged male blowflies.

The tarsal acceptance threshold of nondiapausing adult blowflies rises with age (Stoffolano, 1974b). In addition, Stoffolano (1973) and Rees (1970) noted a decline in the number of functioning peripheral chemoreceptor sensilla among aged flies. Since these receptors are involved in the initiation of feeding (Dethier, 1969), a degenerated receptor system would imply that aged flies are less efficient feeders. Each of the above factors could contribute to reduced feeding in aged flies. In the final analysis, however, this decline in food intake is probably due to several factors, no specific one of which is more important than the others.

Females of both species have a greater total intake of both protein and sugar compared to males (when compared on a μl/day basis). However, when total daily mean intake and total daily mean sugar intake by males are compared to that of females on a μl/mg per day basis, there is no significant difference between the two. Female flies weigh more than males and tend to feed more, though not significantly more. To our knowledge, this study is the first to compare intake values on a μl/mg basis. Previously, Greenberg (1959) found no significant difference in sucrose intake for female compared to male houseflies, and Roberts and Kitching (1974) found that female specimens of *Lucilia cuprina* consumed more sugar and protein compared to males.

In the present study, the finding that female flies consumed significantly more protein compared to males is consistent with previous studies (Greenberg, 1959;

Dethier, 1961; Belzer, 1970), although peaks of protein intake closely correlated with female reproductive cycles (Belzer, 1970; Roberts and Kitching, 1974) were not as apparent in this study. This was probably due to the different environmental conditions at which the flies in this study were reared and tested.

Vitellogenesis in female blowflies is dependent on a protein meal (Rasso and Fraenkel, 1954; Harlow, 1956; Orr, 1964; Belzer, 1970; Bennetová-Řežabová, 1972; Stoffolano, 1974a), while spermatogenesis is independent of feeding in *P. regina* (Cowan, 1932; Mackerras, 1933, Stoffolano, 1974a) and also in other flies (Chaudhury and Ball, 1973). Thus, it would seem obvious that the protein intake of females would be greater than that of males. However, as shown in this study (see Fig. 1B) and others (Dethier, 1961; Belzer, 1970), male blowflies also show a distinct protein selection. A protein meal is necessary for male *P. regina* to develop the reproductive accessory glands and may be required to activate the centers that influence the development of this gland (Stoffolano, 1974a).

The intake of sugar by male and female blowflies is overwhelmingly greater than the intake of protein. Dethier (1961) and Roberts and Kitching (1974) also found that flies having free access to both protein and sugar always consumed greater volumes of the carbohydrate. Dethier (1969) further concluded that the principle food of the blowfly is carbohydrate.

In the present study sugar intake fluctuated markedly. Dethier (1961) attributed these fluctuations to differences in the activity of the fly which correlated with variations in the climate of the laboratory (temperature and humidity were not held constant). Gelperin and Dethier (1967) noted that even under constant temperatures, some fluctuation in sugar intake remains. Our results show that even when temperature and humidity are controlled, these fluctuations in sugar intake still persist.

The second part of the present study dealt with the question of feeding in diapausing flies. The evidence reported in this paper indicates that flies reared in the laboratory under diapause-inducing conditions and exhibiting characteristics indicative of imaginal diapause (arrested ovarian development and hypertrophied fat body) do feed on both protein and sugar.

Reports in the literature concerning insect feeding during diapause are contradictory. Although many insects have been reported to feed while in a reproductive arrest, this activity may be reduced and limited to nonprotein sources and to a "pre-diapause" period (de Wilde, 1954; El-Hariri, 1965; Mitchell and Taft, 1966; Stoffolano, 1968; Hodek, 1971; Brown and Chippendale, 1974).

Mansingh (1971) has divided diapause into three periods and defines "pre-diapause" as a preparatory period when food taken in by the insect is accumulated and stored. These nutrient reserves are used as an energy source to maintain basal metabolic functions during the succeeding "diapause period," when protein synthesis and basal metabolism are reduced. Growth and development are resumed during the "post-diapause" period. Feeding behavior observed for the diapausing flies in this study may represent food intake during a "pre-diapause" period. In both species, and especially *P. terraenovae,* total intake declined during the course of the experiment. This decline could represent the onset of the "diapause" period when feeding is reduced. Forty-day old diapausing specimens of *P. regina* had significantly reduced sucrose intake compared to 40-day old nondiapausing flies

(Stoffolano, 1975). The observation made in the present study that fat body hypertrophy develops in diapausing flies after 12–15 days of feeding suggests that this food taken in by the insect is being accumulated and stored.

In nature, *P. regina* and *P. terraenovae,* entering reproductive diapause in response to shortened fall photoperiods, may continue to feed actively during this time when environmental conditions still permit activity and food sources are still abundant. The adult may feed and even engorge on food to build up metabolic reserves (such as glycogen and fat) to be used as an overwintering energy source. It seems obvious that such a period of feeding when large accumulations of food are set aside for winter should exist (Stoffolano, 1974b).

As already stated, nondiapausing female blowflies require a protein meal to develop their ovaries and therefore will selectively increase their protein intake prior to vitellogenesis. Procedures which disrupt normal ovarian development also disrupt protein feeding (Dethier, 1961). During diapause the ovaries do not develop and yet the evidence presented in this study indicates that the amount of protein taken by diapausing blowflies compared to nondiapausing flies was not significantly different (Table I).

In mosquitoes, the term gonadotrophic dissociation (Swellengrebel, 1929) is commonly applied to any situation where ovaries remain undeveloped in females that have taken a full blood meal (Eldridge, 1966). In such mosquitoes, a large fat body often develops. The function of ovarian diapause may be to divert nutrients from the ovary to the fat body which serves as a storage depot (Spielman and Wong, 1973). In this study, both species of diapausing flies showed fat body hypertrophy. It is not known whether a diapausing female fly taking a protein meal in the fall needs to feed on protein in the spring to develop ovaries.

The feeding behavior of the two species of diapausing blowflies differed in several respects when compared to each other and their nondiapausing counterparts. Intake by diapausing *P. terraenovae* was not significantly different from the nondiapausing population (on a μl/mg per day basis), while diapausing specimens of *P. regina* fed significantly more than nondiapausing flies. The protein intake of both diapausing and nondiapausing populations of flies was not significantly different; differences in sugar intake accounted for the differences seen in total intake. In addition, an enlarged crop full of a clear viscous fluid was seen in diapausing *P. terraenovae* but not in the diapausing *P. regina* population. These differences in feeding behavior could be the result of confounding effects of differences in temperature regimes experienced by the nondiapausing and diapausing *P. regina* populations as already discussed. They may also be related to differences in the ecology of the two species of flies.

Since diapause represents a physiological adaptation evolved to overcome adverse environmental conditions of a particular climatic zone (Mansingh, 1971), the two species may have evolved different overwintering strategies in response to conditions peculiar to their own ecology. *P. regina,* a temperate region species in which diapause is temperature-dependent, may continue to feed while the weather permits. *P. terraenovae* is an arctic species and enters dispause apparently independent of the temperature. Other cues, such as the enlarged crop, may control feeding behavior in this species. Knowledge of the winter biology of these two species is limited, and consequently further study on the behavior of these flies in nature is needed.

Intake is more affected by the age of the fly in the diapausing populations than in the nondiapausing population of flies. This effect was greater for *P. terraenovae* than for *P. regina*. In diapausing specimens of *P. terraenovae,* the decline in feeding was coincident with the appearance of fat body hypertrophy and a full crop during days ± 14. As the abdomen of the diapausing fly becomes distended due to fat hypertrophy, and in the case of *P. terraenovae* a full crop, body wall stretch receptors may send impulses to the central nervous system resulting in a decline of food intake. This inhibition may occur at the central level of the nervous system rather than at the peripheral level, since the tarsal acceptance threshold of diapausing flies is not different from that of nondiapausing flies (Stoffolano, 1975). Thus it may *not* be the same system of negative feedback operating in the nondiapausing replete fly to inhibit further feeding (Gelperin, 1971) as is operating in the diapausing fly to reduce feeding. However, rather than a real aging effect, which is irreversible, these other factors may have resulted in the decline in food intake and thus the statistically greater aging effect seen in diapausing *P. terraenovae* compared to the nondiapausing populations. Forty-day old diapausing specimens of *P. regina*, removed from diapause conditions and held at nondiapause conditions for 13 days, show an increase in food intake comparable to that of 40-day old nondiapausing flies (Stoffolano, 1975).

The feeding behavior of these flies in nature before, during, and after the overwintering diapause is not known. However, our results are consistant with the idea that diapause in *P. regina* and *P. terraenovae* may occur in three stages as outlined by Mansingh (1971). The first stage, characterized as a preparatory stage, is accompanied by trophic levels of metabolism and arrested ovarian development. Though the insect continues to feed, due to reduced levels of protein synthesis and the occurrence of gonadotrophic dissociation, nutrients are not metabolized along reproductive pathways but along so called "diapause pathways". This accumulation of nutrient reserves results in fat body hypertrophy which may act on the central nervous system *via* stretch receptors to inhibit feeding. During the second stage of diapause, the insect ceases feeding, and respiration drops to the level of basal metabolism (Slama, 1964). The first stage of diapause or prediapause would be synchronized to occur with the still favorable environmental conditions existing in the fall. The insect could take advantage of these conditions to feed and store this food as reserves to be used later during the severe conditions of freezing temperatures and lack of food sources associated with winter. The lower rate of metabolism during the winter presumably leads to a show utilization of food reserves. As the food reserves are utilized, neural inhibition may be released and the postdiapause adult may feed again when warmer temperatures in the early spring permit activity.

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Summary

1. The long-term intake of protein and sugar by nondiapausing and diapausing male and female *P. regina* and *P. terraenovae* was determined using a two-choice apparatus with statistical analysis of the results (both μl/day and μl/mg per day).

2. Results of studies using nondiapausing *P. regina* and *P. terraenovae* were found to be similar. Intake was significantly influenced by the age of the fly, aged flies tending to feed less. Males and females exhibited similar trends in food intake as they aged. Sugar intake by male and female blowflies was significantly greater than protein intake. Female flies consumed significantly more protein than males. However, on a μl/mg basis there was no significant difference in either sugar intake or total intake of protein plus sugar between male and female flies.

3. Diapausing flies were found to feed on both sugar and protein, although sugar intake was significantly greater than protein intake. Protein intake by both species of diapausing flies did not differ significantly from that of nondiapausing flies. However, diapausing specimens of *P. regina* had a significantly greater sugar intake than the nondiapausing *P. regina* population; while diapausing specimens of *P. terraenovae* fed significantly less on sugar than the nondiapausing *P. terraenovae*. These results were the same when considered on a μl/mg basis. In the *P. terraenovae* population, intake was found to be more influenced by age among diapausing flies compared to nondiapausing flies.

4. An analysis of covariance showed that the effect of fly weight on intake was not significant.

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THE SHAPING OF THE ORNAMENTED FERTILIZATION MEMBRANE OF *COMANTHUS JAPONICA* (ECHINODERMATA: CRINOIDEA)

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The fertilization membrane of echinoderms typically has an outer surface that appears smooth by light microscopy. However, some crinoid echinoderms are exceptional, since they have a fertilization membrane covered with conspicuous protuberances (Mortensen, 1920a, 1920b, 1937, 1938; Dan and Dan, 1941). Although the protuberances were originally thought to be spines, more recent work has shown them to be ridges (Holland and Jespersen, 1973). These ridges are produced and shaped by the female germinal cell acting without the aid of ovarian follicle cells; in this respect, the crinoid fertilization membrane differs fundamentally from egg envelopes bearing surface processes produced and shaped by the follicle cells, as in some teleosts (Wourms and Sheldon, 1976) and chitons (Richter, 1976). According to Mortensen (1920a), the main function of the ridges on the crinoid fertilization membrane is to reduce the sinking rate and facilitate drift dispersal of the planktonic eggs and embryos.

In their light microscopic study of *Comanthus japonica*, an unstalked crinoid, Dan and Dan (1941) showed that surface topography is transferred from the oocyte to the fertilization membrane. Light microscopy, however, could not convincingly demonstrate how this transfer is accomplished. Therefore, in the present investigation, the formation of the fertilization membrane of *Comanthus japonica* is described by electron microscopy. The results leave little doubt that oocyte topography acts as a first template, shaping a pattern of jelly on the surface of the unfertilized egg; this jelly, in turn, acts as a second template, shaping the pattern of ridges on the outside of the fertilization membrane. The morphogenetic role of the jelly is corroborated in the present study by data showing that a smooth surfaced fertilization membrane can be produced if the jelly is first removed from the living egg.

Materials and Methods

Specimens of *Comanthus japonica* were collected in Koaziro Bay, Kanagawa Prefecture, Japan, from a few days to a few hours before spawning; the date and even the hour of spawning can usually be predicted in advance (Dan and Kubota, 1960). Animals were held individually in dishes of running sea water at the nearby Misaki Marine Biological Station. Oocytes were obtained by dissecting ovaries, and ripe eggs were obtained from females spawning in the laboratory. For insemination, one drop of undiluted sperm from a testis of a male was mixed rapidly with several thousand eggs in 100 ml of sea water at 22 ± 1° C.

For transmission electron microscopy (TEM), oocytes and eggs were fixed for 90 min at room temperature in 3% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.3) with 0.45 M sucrose. Specimens were rinsed at room temperature in the same buffer with 0.45 M sucrose before post-fixation for 60 min on ice in 1% osmic acid in the same buffer with 0.45 M sucrose. This procedure, which is illustrated by Figures 4, 11, 13, and 21, gave good cytoplasmic fixation, but preserved the egg jelly poorly or not at all. The jelly was preserved for TEM, with less than optimal fixation of cell membranes, by a modification of the method of Behnke and Zelander (1970). Oocytes and eggs were fixed for 90 min at room temperature in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.5) with 1% alcian blue 8GN (B408AX440) and 0.35 M NaCl; the NaCl was added immediately before use. Specimens were rinsed at room temperature in the same buffer with 0.5 M NaCl, but the postfixation of Behnke and Zelander (1970) was omitted, since it removed the jelly. This TEM fixation with alcian blue is illustrated by Figures 3, 7, 8, 14, 16, and 17. All TEM specimens were rapidly dehydrated in an ethanol series, transferred to propylene oxide and embedded in Epon. Contrast of silver sections was enhanced with uranyl acetate and lead citrate.

For scanning electron microscopy (SEM), oocytes and eggs were fixed several days at room temperature in a solution of 2% glutaraldehyde in dilute (77%) sea water; this fixative was approximately isotonic with full strength sea water. The procedure removed the jelly, as illustrated by Figure 10. The jelly was preserved for SEM by the modified method of Behnke and Zelander (1970), which has been described in the preceding paragraph. This SEM fixation with alcian blue is illustrated by Figures 1, 2, 5, 6, 9, 12, and 15. All SEM specimens were rapidly dehydrated in an ethanol series, transferred through Freon 113 and dried by the Freon critical point method. The dried oocytes and eggs were mounted on stubs with double-stick tape (Scotch Brand), rotary coated with a mixture of gold and palladium (60:40) and viewed in a Cambridge S4 scanning electron microscope.

Jelly was removed from living eggs by a 30-min incubation in calcium-free sea water prepared according to Harvey (1956, p. 156). Such eggs, even if returned to normal sea water, did not respond to insemination; however, they did activate after treatment in ionophore by the method of Steinhardt and Epel (1974). For activation, 5 μl of a 5 mM solution of ionophore A23187 (Eli Lilly Co.) in dimethylsulfoxide was mixed rapidly with several hundred eggs in 5 ml of sea water (with or without calcium) at 22 ± 1° C. Appropriate controls of dimethylsulfoxide without ionophore never resulted in egg activation.

RESULTS

The dented oocyte

Throughout the last week of oogenesis in *Comanthus,* the oocyte surface (Fig. 1) is dented with several hundred pits, each about 10 μ across by 10 μ deep. Every pit contains a conspicuous mass of extracellular jelly. This jelly appears to be produced by the oocyte and is rich in nonsulfated acid mucopolysaccharides (Holland, Grimmer and Kubota, 1975). Each mass of jelly will be termed a jelly clump and should not be confused with the copious but diffuse mucus (probably of epidermal origin), which briefly surrounds the eggs after spawning (Holland and Grimmer, 1975). A jelly clump, as seen at relatively low magnification, consists of interconnected strands and globules (Figs. 2 and 3). Some of the strands are closely apposed to the oocyte's plasma membrane at the bottom of the pit (Fig. 3, single arrow) and appear to hold the jelly clump in place. At higher magnification, the jelly has a finely granular consistency.

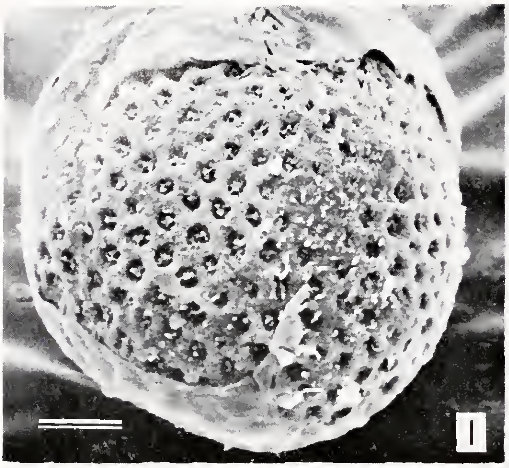
The plasma membrane of the oocyte is folded into ridges called microplicae (as defined by Andrews, 1976). The microplicae, which are too small to be visible in Figure 1, occur on all parts of the oocyte surface, whether undented (Fig. 2) or dented (as determined by serial sectioning). In cross section, each microplica is about 0.5 μ high and just over 0.1 μ wide and is capped with a thin layer of extracellular material several hundred Ångstroms thick (Fig. 4, arrow). The cortical cytoplasm of the oocyte includes cortical granules (Fig. 3, twin arrow), which are membrane-bound organelles about 1 to 3 μ diameter. The finely granular contents of each cortical granule are divisible into irregular dense patches and a less dense matrix (Fig. 4).

The unfertilized egg

Oocytes differentiate into eggs prior to spawning. The unfertilized egg (Figs. 5–7) no longer has the pitted surface that characterized the oocyte. However, the jelly clumps remain distributed over the egg surface in the same pattern they had on the oocyte (Figs. 5 and 6). Each clump probably indicates the position of a former pit on the oocyte surface. In comparison to the jelly clumps of the living egg (Fig. 18), those of the fixed egg are somewhat shrunken (Fig. 5). As seen at higher magnification the egg jelly has a substructure suggestive of closely packed tubules (Fig. 8). Each tubule has a dense wall and a lucent core. Tubule diameters are roughly 250 Å, and tubule lengths may reach at least several thousand Ångstroms; there is no apparent order in the arrangement of the individual tubules. The jelly clumps seem held in place on the egg surface (Fig. 7) by close association with the outside of a vitelline coat. The vitelline coat (Figs. 8, 11, and 21) is a moderately dense layer of extracellular material about 100 to 150 Å thick, which closely follows the plasma membrane around the circumference of the egg. Most of the vitelline coat suddenly appears while the oocyte is differentiating into the egg a few hours before spawning. It is not known whether the vitelline coat incorporates any of the extracellular material that capped the oocyte's microplicae. Instead of microplicae, the egg surface bears scattered microvilli up to 0.5 μ long (as demonstrated by serial sections). Beneath the plasma membrane of the egg, cortical granules are located in the cortical cytoplasm (Fig. 7).

The cortical reaction and fertilization membrane formation

Exocytosis of cortical granules begins about 45 sec after insemination of the *Comanthus* egg at the presumed point of sperm entry; the cortical reaction then spreads over the egg surface to the opposite pole in about 60 sec (Dan and Dan, 1941). On the egg in Figure 9, the cortical reaction started at the far left and progressed roughly halfway over the surface by the instant of fixation. In surface view, the leading edge of the reaction is obscured by the jelly clumps in Figure 9, but can be seen on eggs from which the jelly has been removed (Fig. 10). Just behind the leading edge, the newly formed fertilization membrane is ridgeless; then, some 25 μ behind the leading edge, the erection of the ridges begins.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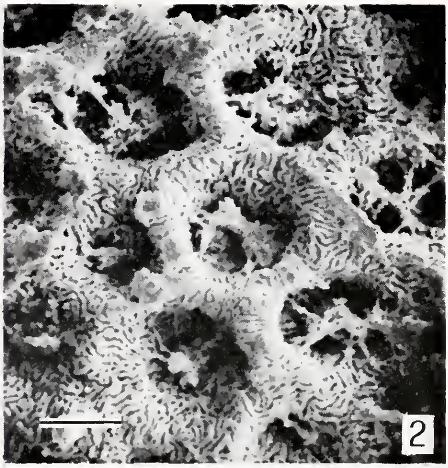
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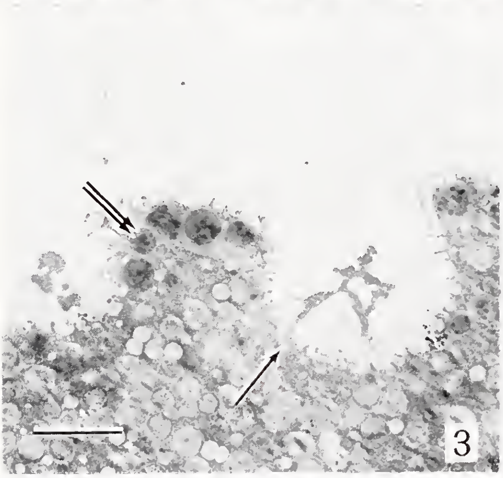
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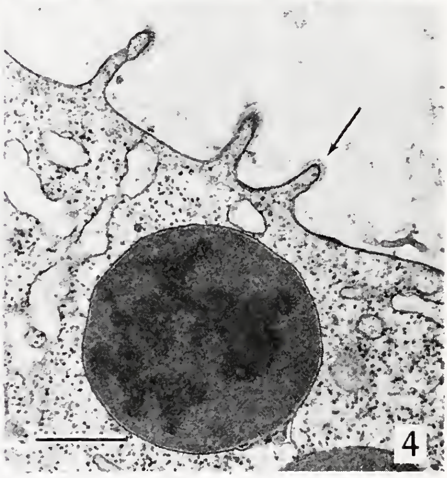
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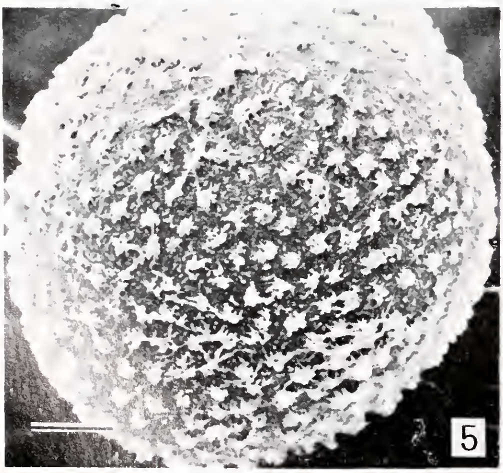
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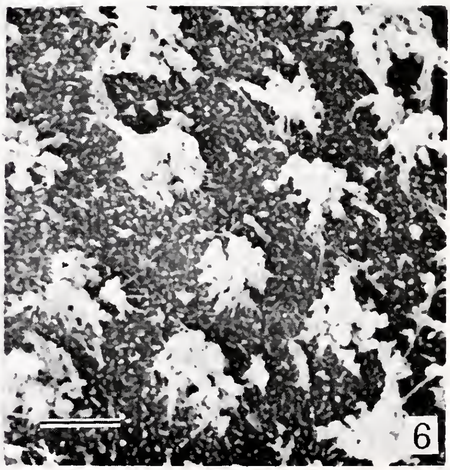
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The exocytosis of a cortical granule at the leading edge of the cortical reaction is shown in Figure 11. Erupted cortical granule material is restrained by and adheres to the inside of the vitelline coat, which is pushed away from the plasma membrane of the egg. As in sea urchins (reviewed by Schmekel, 1975), the fertilization membrane of *Comanthus* is a composite of vitelline coat and cortical granule material. However, in comparison to the fertilization membrane of sea urchins, that of *Comanthus* includes on the order of a hundred times as much cortical granule material.

As exocytosis begins, the cortical granule contents increase in volume and decrease in density (Fig. 11). For several seconds thereafter, the dense patches of the cortical granule material can still be distinguished from the less dense matrix (Figs. 11 and 13). By the time ridge elevation is just beginning, the fertilization membrane has resolved itself into a thin outer component (between the arrows in Fig. 13) and a thick inner component. The outer component has a dense, granular consistency and is roughly 0.1 μ thick; this thickness is nearly ten times that of the vitelline coat, and the outer component presumably includes a large amount of cortical granule material in addition to the vitelline coat. A very thin layer, possibly the vitelline coat, reappears at the outer surface of the outer component 20 min after insemination (Holland and Jespersen, 1973). The inner component of the fertilization membrane, as ridge erection begins, is roughly 2 μ thick and is made up of fibrogranular material derived from the cortical granules (Fig. 13). Microvilli up to 2 μ long are extended from the egg surface as cortical granule exocytosis is taking place; at first, these microvilli project into the substance of the fertilization membrane (Fig. 14).

Several seconds after the start of the cortical reaction, ridges begin to arise on the surface of the fertilization membrane (Fig. 10). These ridges appear between adjacent jelly clumps and form a pattern of hexagons with an occasional pentagon (Figs. 9 and 12). Each polygon of ridges outlines a depression that I will call a facet. Within each facet, a jelly clump remains in close association with the outside of the fertilization membrane (Fig. 14). During the next minute or two, the ridges grow progressively higher, reaching a maximum height of about 15 μ (Figs. 15 and 16). As maximum ridge height is attained, the inner component of the fertilization membrane has become distinctly fibrous (Fig. 17).

The simplest explanation of ridge formation is that the jelly clumps act as physical restraints, holding down the facets, while the ridges are able to rise only in the spaces between adjacent jelly clumps. One can speculate that the vitelline

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FIGURE 1. A dented oocyte with numerous surface pits, each containing a jelly clump (remnants of ovary adhere at top and bottom). The scale line is 30 μ.

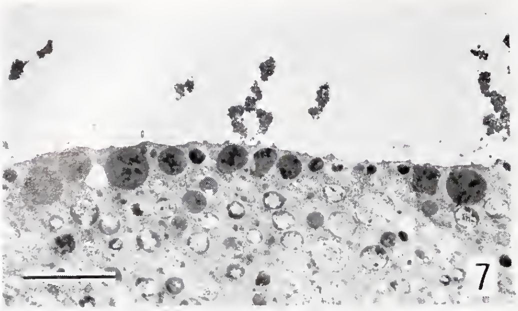
FIGURE 2. Surface detail of a dented oocyte with jelly clumps in pits. The scale line is 7 μ.

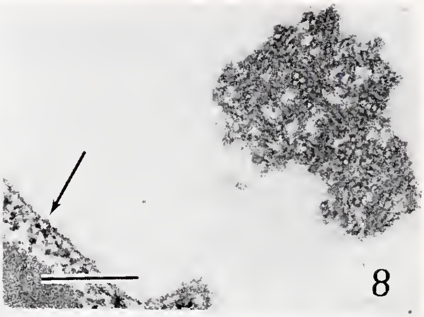
FIGURE 3. The periphery of a dented oocyte, with pits containing jelly clumps at left and center. The single arrow indicates close association between jelly and the oocyte surface. The twin arrow points to a cortical granule. The scale line is 5 μ.

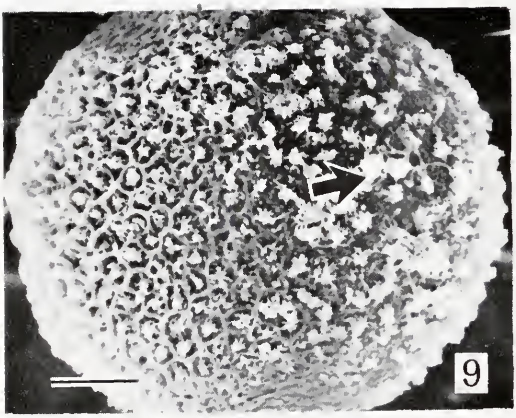
FIGURE 4. Details at the periphery of a dented oocyte. The arrow indicates the layer of extracellular material capping a microplica. The conspicuous organelle in the cytoplasm is a cortical granule. The scale line is 0.7 μ.

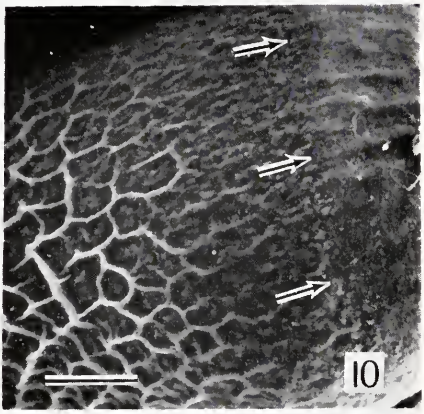
FIGURE 5. An unfertilized egg covered with jelly clumps, which have the same distributional pattern they had on the oocyte. The scale line is 30 μ.

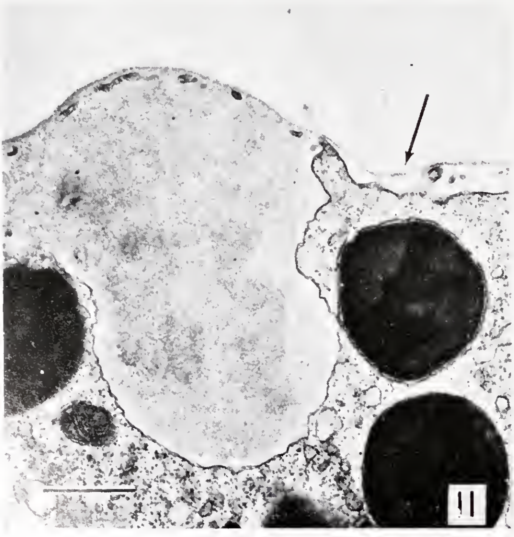
FIGURE 6. Surface detail of an unfertilized egg with jelly clumps. The scale line is 7 μ.











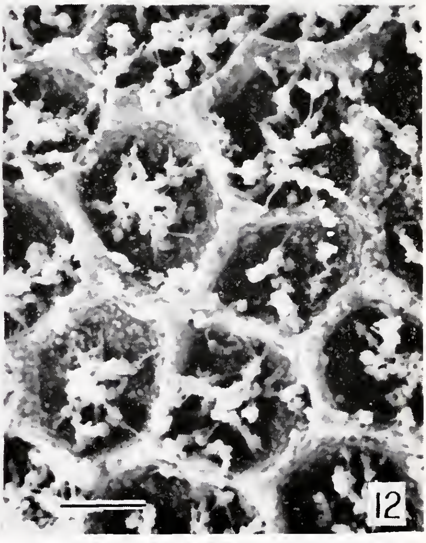


FIGURE 7. The periphery of an unfertilized egg. Portions of jelly clumps appear at left, center and right. Cortical granules are conspicuous just beneath the plasma membrane. The scale line is 5 μ.

coat is elastic enough to permit ridge erection, and that adhesion between each jelly clump and the underlying vitelline coat inhibits lateral spreading of the facet floor. The force causing the ridges to rise is not known with certainty, but could well be osmotic.

As ridge formation is in progress, the inside of the fertilization membrane separates from the underlying plasma membrane of the egg, leaving a perivitelline space several microns wide (Fig. 16, arrow). During fertilization membrane elevation, the microvilli of the egg are pulled out of the substance of the fertilization membrane and are left projecting into the perivitelline space. No trace of a hyaline layer is detectable in the perivitelline space. At 4 min after insemination, jelly clumps are still located in the facets of the fertilization membrane. It is not known whether the jelly ultimately dissolves, since older fertilization membranes were not prepared with alcian blue added to the fixative.

Light microscopic observations of living eggs

If, as the electron microscopy indicates, the presence of jelly clumps on the egg is necessary for ridge formation on the fertilization membrane, then insemination of a jellyless egg should result in production of a ridgeless fertilization membrane. The jelly clumps of the living egg (Fig. 18) can readily be removed by exposure to calcium-free sea water (Fig. 20), a treatment that does not remove the vitelline coat (Fig. 21). At this point there is a technical difficulty, since eggs treated in calcium-free sea water and then inseminated never undergo a cortical reaction, even when returned to normal sea water prior to insemination; the reasons for this failure have not been studied, although lack of jelly could well prevent the sperm from binding to or entering the eggs. Fortunately, eggs treated in calcium-free sea water can undergo a cortical reaction after exposure to ionophore A23187; the reaction occurs whether or not calcium is present in the medium during ionophore administration.

When control eggs, which have not been dejellied in calcium-free sea water, are treated with ionophore A23187, there is a latent period of about 45 sec, and then cortical granule exocytosis occurs simultaneously over the entire surface of the egg. During the 90 sec after exocytosis, a fertilization membrane with surface ridges is

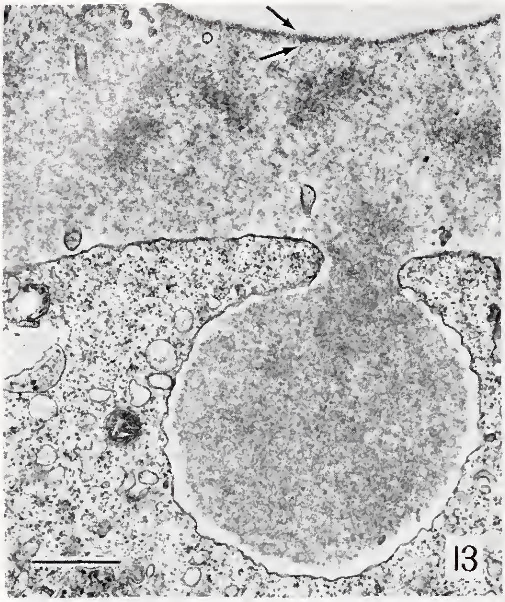
FIGURE 8. Detail at the periphery of an unfertilized egg. The plasma membrane (poorly preserved) is covered with a vitelline coat (arrow). A portion of a jelly clump is at the right. The scale line is 0.5 μ.

FIGURE 9. An egg fixed 70 sec after insemination. The cortical reaction began at left center and, at the instant of fixation, was spreading in the direction of the arrow. At the right, the surface is comparable to that of the unfertilized egg; at the left, the ridges of the fertilization membrane are rising among the jelly clumps. The scale line is 30 μ.

FIGURE 10. An egg fixed 70 sec after insemination with the cortical reaction spreading from left to right. The jelly has been removed to show the leading edge of the cortical reaction (indicated by the arrows). The scale line is 25 μ.

FIGURE 11. Detail of the periphery of an inseminated egg at the leading edge of the cortical reaction, which is proceeding from left to right. The vitelline coat (arrow) restrains the erupting cortical granule material. The scale line is 1 μ.

FIGURE 12. Surface view of the fertilization membrane where the ridges are just beginning to form. The ridges outline polygonal facets in which the jelly clumps are located. The scale line is 7 μ.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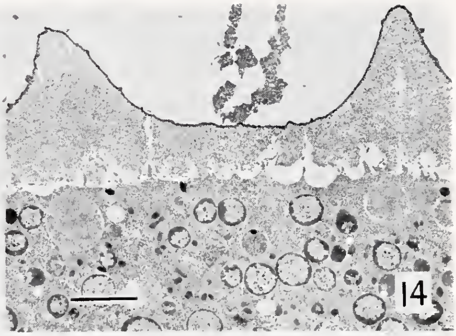
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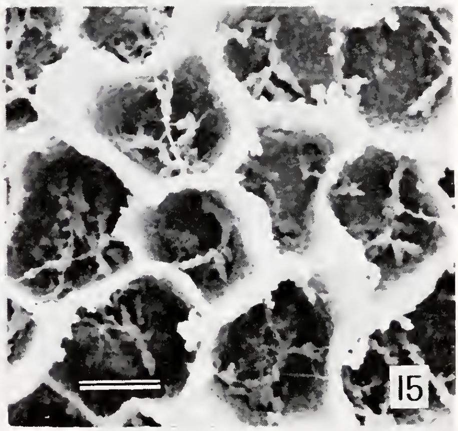
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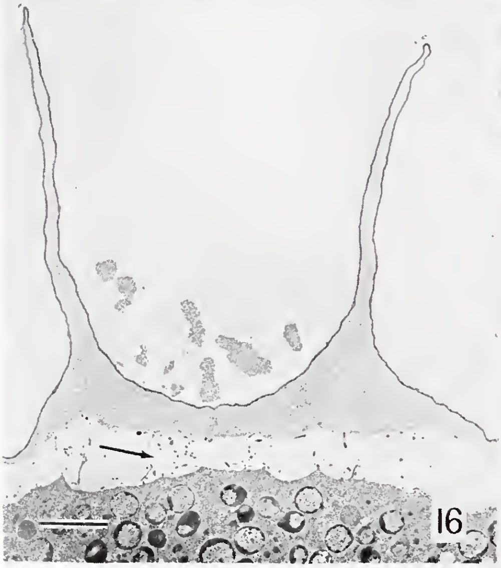
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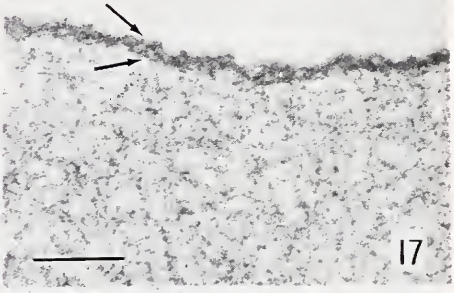
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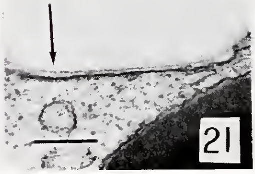
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produced; such a fertilization membrane appears structurally identical to one elicited by insemination both by light microscopy (Fig. 19) and by electron microscopy.

When eggs are de-jellied in calcium-free water for 30 min, returned to normal sea water for 10 min (to prevent excessive swelling of the fertilization membrane) and then activated with ionophore A23187, a ridgless fertilization membrane is produced. This smooth fertilization membrane begins to appear about 45 sec after ionophore administration and, during the next 15 sec, attains maximum thickness (Fig. 22). The production of a smooth fertilization membrane after removal of the jelly strongly supports the argument that the pattern of jelly clumps on the egg surface is a proximate cause of the pattern of ridges on the fertilization membrane surface.

Discussion

The ridge pattern on the fertilization membrane of *Comanthus* can be traced back, *via* the jelly clump pattern on the egg, to a pit pattern on the oocyte surface. How the oocyte initiates and maintains its pitted surface topography remains a mystery. Just when the pits are forming, cytoplasmic fibers temporarily become conspicuous in the oocyte cytoplasm (Holland, Grimmer and Kubota, 1975). It is possible that these fibers could be involved in pit formation; unfortunately, they

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Figure 13. Detail of the periphery of an inseminated egg where the ridges are just beginning to form on the fertilization membrane. The fertilization membrane has resolved itself into a thin, dense outer component (between the arrows) and a thick, less dense inner component. The scale line is 1 μ.

Figure 14. The periphery of an inseminated egg in a region comparable to the far left in Figure 10. A jelly clump (top center) occupies a facet of the fertilization membrane. The scale line is 3 μ.

Figure 15. Surface of the fertilization membrane of an inseminated egg between 1 and 2 min after the passage of the cortical reaction. The surface ridges have attained their maximum height of about 15 μ, and jelly clumps still occupy the facets. The scale line is 7 μ.

Figure 16. The periphery of an inseminated egg between 1 and 2 min after the passage of the cortical reaction. A jelly clump occupies a facet bounded by fully formed ridges. The fertilization membrane has lifted away from the cell surface, leaving a perivitelline space (arrow). The scale line is 3 μ.

Figure 17. Detail of the fertilization membrane between 1 and 2 min after the cortical reaction. The relatively thin, dense outer component (between the arrows) overlies the thick, fibrous inner component. The scale line is 0.3 μ.

Figure 18. A living, unfertilized egg photographed uncompressed under phase contrast (as were the eggs in Figs. 19, 20 and 22). The jelly clumps are conspicuous. The scale line (which is also applicable to Figs. 19, 20 and 22) is 100 μ.

Figure 19. A living egg 2 min after insemination. The fertilization membrane with fully formed ridges is conspicuous. Living eggs 2 min after administration of ionophore A23187 appear exactly the same as this inseminated one.

Figure 20. A living, unfertilized egg 3 min after exposure to calcium-free sea water. The jelly clumps are dissolving and will have vanished completely in a few minutes more.

Figure 21. Detail of the periphery of an unfertilized egg after 30 min in calcium-free sea water. The vitelline coat (arrow) is still present. The scale line is 0.3 μ.

Figure 22. A living egg, de-jellied in calcium-free sea water for 30 min, returned to normal sea water for 10 min and then exposed to ionophore A23187 for 10 min. A ridgeless fertilization membrane has been produced.

have been studied only by light microscopy. The maintenance of the dented surface is not correlated with a conspicuous framework of microtubules of microfilaments in the cortical cytoplasm of the oocyte (Holland, unpublished). Indeed, no microtubules were detected there by TEM (although the same fixation preserved the microtubules of the meiotic spindles), nor were microfilament networks well developed. There is some evidence that cell surface protein may play a role in maintenance of the dented topography of the oocyte, since dented oocytes become smooth 4 min after being placed in sea water containing 0.01% protease (Sigma, type VI from *Streptomyces griseus*) (Holland, unpublished). The protease-smoothed oocytes lose their microplicae, but remain surrounded by jelly clumps. It is not known if the protease acts on the extracellular layer capping the microplicae, on proteins of the plasma membrane, or on both. It is also possible that the protease ultimately has a transmembrane effect on the cortical cytoplasm of the oocyte. Recent work on vertebrate cells *in vitro* also indicates that cell surface protein profoundly influences cell shape (Mallucci and Wells, 1976; Yamada, Yamada and Pastan, 1976).

The jelly clump on the egg consists of tubules approximately 250 Å in diameter. Light microscopic histochemistry indicates that the jelly clumps are rich in nonsulfated acid mucopolysaccharide (Holland, unpublished); therefore, each tubule probably has a polyanionic surface. Such polyanionic tubules could be cross linked with one another by calcium ions, which would explain why the jelly clumps so readily dissolve in calcium-free sea water. The polyanionic nature of the tubules is further indicated by their stabilization when the cationic dye, alcian blue, is added to fixative solutions. The substructure of tightly packed tubules might give the jelly clump as a whole a relatively firm consistency, well suited for holding down the facet floors during fertilization membrane formation. The tubules of the jelly clumps of *Comanthus* appear to differ chemically from the proteinaceous "microtubule mimics" discussed by Wourms and Sheldon (1976, p. 360).

The fertilization membrane of *Comanthus* is probably made up chiefly of proteins derived from the vitelline coat and from structural material originating from the cortical granules. The vitelline coat is soluble in dithiothreitol and is presumably rich in disulfide-linked proteins (Holland, 1976a), while the material derived from the cortical granules gives positive histochemical reactions for protein (Holland, unpublished). At present, nothing is known of the biochemical reactions that bring about the formation of the dense outer component and the fibrous inner component of the fertilization membrane of *Comanthus*.

The maximum height of the ridges is probably held to about 15 μ by one or more of the following: a limited amount of stretch in the vitelline coat, a loss of osmotically active substances from the inner component, or an increase in the structural integrity of the inner component. The long microvilli erected concomitantly with cortical granule exocytosis presumably do not push up the ridges on the fertilization membrane, since these microvilli do not exceed a few microns in length and are as abundant beneath the facets as beneath the forming ridges (Fig. 16). It seems more likely that such microvilli form at exocytosis to help accomodate excess membrane resulting from the sudden fusion of cortical granule membranes with the overlying plasma membrane, a problem discussed by Eddy and Shapiro (1976).

The eggs of *Comanthus* are shed directly into the sea water, which is probably the commonest and most evolutionally primitive mode of reproductive behavior among crinoids (Mortensen, 1937; Holland, 1976c). In crinoids for which reproduction has been studied, the spawning of eggs into the sea water is always followed by the production of an ornamented fertilization membrane (Mortensen, 1920a, 1920b, 1937, 1938; Dan and Dan, 1941). During evolution, crinoids spawning eggs into the sea water are presumed to have given rise to crinoids brooding eggs on or in the body of the mother (Holland, 1976c). The evolution of brooding is associated with the loss of ridges from the fertilization membrane, which is relatively smooth in brooding species (Clark and Clark, 1967, pp. 168 and 636). For an external brooder, ridges on the fertilization membrane would probably be worse than useless, since they would interfere with adherence to the mother's epidermis; for an internal brooder, such ridges would needlessly take up space in the mother's brood pouches.

It should be added that some ophiuroid echinoderms of the genus *Ophiocoma* have ornamented fertilization membranes (Mortensen, 1937), apparently of the type described here for *Comanthus*. The significance of this peculiar coincidence is obscured by the present uncertainty about the phylogenetic relationships among the living echinoderm classes (Ubaghs, 1967).

In the course of normal development, the ultimate fate of the fertilization membrane is dissolution, presumably under the influence of a proteolytic hatching enzyme produced by the embryo (Dan and Dan, 1941). Dissolution begins from the inner surface of the membrane about 13 hr after fertilization, and the embryo hatches out about 2 hr later (Holland, 1976b).

The present investigation is affectionately dedicated to Drs. Jean and Katsuma Dan, who helped show me the way. I am also deeply indebted to Director Hiroshi Terayama and Assistant Director Shonan Amemiya for their kind hospitality and for their generous loan of facilities at the Misaki Marine Biological Station of the University of Tokyo. This work was supported in part by U.S.P.H.S. Biomedical Sciences Support Grant No. R-07011. Ms. Ellen Flentye gave competent assistance with the SEM, and the manuscript was criticized by Dr. David Epel, Ms. Linda Holland, Dr. Mia Tegner and Dr. Victor Vacquier.

SUMMARY

1. This is the first electron microscopic description of fertilization membrane formation in a crinoid echinoderm.

2. The fertilization membrane is a composite structure consisting of a thin vitelline coat plus a thick layer of material originating from the cortical granules.

3. The fertilization membrane elevates from the plasma membrane, leaving a perivitelline space several microns wide; no trace of a hyaline layer appears in this space.

4. By 2 min after the start of cortical granule exocytosis, ridges about 15 μ high are erected on the surface of the fertilization membrane. The ridge pattern is

determined by the egg jelly which is divided into several hundred clumps on the egg surface; each jelly clump apparently acts as a physical restraint on the fertilization membrane surface immediately beneath, and ridges can rise only between the jelly clumps.

5. The morphogenetic role of the egg jelly is corroborated by observations on living eggs; a ridgeless fertilization membrane can be produced if the jelly is first removed from the egg surface.

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GENETIC ASPECTS OF LARVAL GROWTH UNDER REDUCED SALINITY IN *MYTILUS EDULIS*

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Although the ecology and physiology of adult *Mytilus edulis* have gained considerable attention over the years (reviewed in Bayne, 1976), much less information is available for the pelagic larval stage. Compared to some other invertebrate larvae, *Mytilus* larvae spend a relatively long period (three to several weeks) in the plankton. The ability of larvae to grow, successfully settle and develop into adults depends on their response to the range of temperature, salinity and food conditions during this planktonic stage. Fortunately, work by Bayne (1964, 1965, 1970) has established techniques for the routine culture of mussel larvae and provides basic information on the effects of food, temperature and salinity on larval growth. Brenko and Calabrese (1969) give some data on the combined effects of temperature and salinity on larval growth. Similar information is available for a number of bivalve species (Davis and Calabrese, 1964; Calabrese, 1969; Lough and Gonor, 1973).

Measuring larval response under different conditions determines the effect environmental parameters can have on observed variation in quantitative traits, such as growth. A factor not taken into account in studies on marine invertebrate larvae is the effect genetic variation and its interaction with environmental conditions have on larval growth. This is usually ignored or at best treated as within-population variation by using a pool of larvae derived from several sets of parents. Partitioning observed variation into its components is necessary for a more complete understanding of the role genetic variation plays in determining phenotypic variation in various traits of individuals in a population. The relationship between phenotypic variation and genetic variation is fundamental to studying the adaptation and evolution of organisms with reference to particular environmental situations (Lewontin, 1974).

A genetic component of variation and its interaction with the salinity environment can be estimated for larval growth in a population of *Mytilus*. This involves raising individual families of larvae of known parentage under different salinity conditions. Breeding techniques have now been used in several species as a means of investigating quantitative variation in marine organisms (Chapman, 1974; Doyle, 1974; McLaren, 1976; Newkirk, Haley, Waugh and Doyle, 1977). *Mytilus edulis* is particularly suitable, not only because of its well-studied biology, but because individuals release large amounts of gametes, allowing for multiple matings and more efficient experimental designs.

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MATERIALS AND METHODS

Field collections

Adult specimens of *M. edulis* were collected from Ostrea Lake, a shallow, enclosed bay with a narrow opening near the mouth of Musquodoboit Harbour, Nova Scotia. An idea of the salinity and temperature characteristics of this habitat is given in Figure 1 (data provided by L. MacLeod, Nova Scotia Department of Fisheries, Halifax, personal communication) Natural spawning usually begins here in early June, when the water temperature reaches 14–15° C (MacLeod, personal communication). Mussels described in these experiments were collected a few months prior to natural spawning and maintained in running sea water in the laboratory. Gamete maturation was accelerated by increasing the water temperature in steps over a few weeks from 4° C to about 12° C. During this period the mussels were fed regularly a suspension of algae (mostly *Tetraselmis* sp.). Spawnable individuals were obtained well before those in the natural population.

Raising the larvae

Larvae were cultured using the techniques of Bayne (1965). Spawning was induced by placing individuals in heated (23° C), filtered sea water. Usually spawning began within half an hour. Eggs from each female were pipetted and suspended into individual beakers of filtered sea water (from a 5 μm filter). These were then divided equally among several 1000 ml beakers (depending on the experimental design). The eggs were fertilized by adding about 1 ml of sperm suspension and evenly mixing it with the eggs. Beakers of fertilized eggs were held at 16° C for the first 48 hours of development.

Density of the swimming veliger larvae was estimated 48 hours after fertilization and new beakers were set up at about 20 larvae/ml in 900 ml of sea water. The larvae were fed a suspension of a single-celled alga, *Isochrysis galbana*, at a concentration of about 80 cells/μl in each beaker. Every 48 hours the water in each beaker was passed through a 44 μm nitex screen to retain the larvae. Each beaker was rinsed with distilled water followed by sea water. The larvae were then resuspended in filtered sea water with sufficient *Isochrysis* to give the desired cell concentration. Antibiotics were used, with Penicillin-G and Streptomysin sulfate at concentrations of 50.8 mg/liter and 22.2 mg/liter, respectively. Between such changes, the beakers of larvae were maintained in a 16° C temperature-controlled room in the dark. Complete randomization was used at all stages of the experimental procedure. Larval growth was estimated by sampling larvae from each beaker at various times after fertilization and measuring the longest axis of 30 or 40 individuals on the projection screen of an inverted microscope.

Experiments

Two salinity experiments were conducted to investigate the effect of low salinity on larval growth. The first experiment involved three males crossed with a single female. The larvae were pooled 48 hours after fertilization to give a homogenous mixture of the three families. Four beakers of this pooled group were set up at a salinity of 30‰, while two beakers were reduced to 18‰ by adding distilled water (salinities in all experiments were controlled within less than a part per thousand). Larval length was measured at 6, 12, and 18 days after fertilization.

In a second salinity experiment, a pooled group of larvae was produced from two males crossed to a single female. Three salinity treatments were used (30‰, 16‰, and 11‰) with two replicate beakers within each salinity. The two low salinity groups were first reduced to 20‰ three days after fertilization and on the fourth day reduced to the treatment levels of 16 and 11‰. An initial mean larval length was determined three days after fertilization for the pooled group. Subsequent measurements were made 6, 11, 15, and 19 days after fertilization.

Pooling families of larvae is an efficient way of estimating the response of a population to variations in salinity, but this procedure confounds genetic information with the unexplained within-treatment variation. Two experiments were designed to determine the genetic contribution to variation in larval growth and its interaction with salinity. The first experiment was a factorial mating in which six males and six females were crossed in all possible combinations. Each of the resulting 36 families were raised at two salinities (30‰ and 12‰) for 16 days, when mean larval length was measured. Eight males were paired at random with eight females in a second genetic experiment. These eight unrelated, single-pair families were raised under three salinity conditions (30‰, 18‰, and 12‰). After 16 days of growth, mean length was estimated from a sample of larvae from each beaker.

The components of variation in each genetic experiment were estimated by factorial analyses of variance (Sokal and Rhlof, 1969). The first experiment was designed as a three-way, mixed model ANOVA with males and females as random

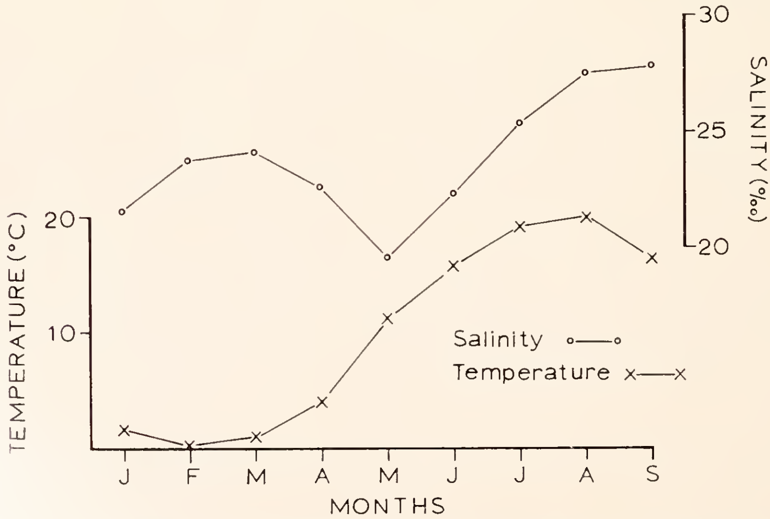

FIGURE 1. Monthly mean temperature and salinity for 1975 taken from a depth of 3 meters in Ostrea Lake, Nova Scotia. Standard errors for each month are all less than 3° C and 1‰.

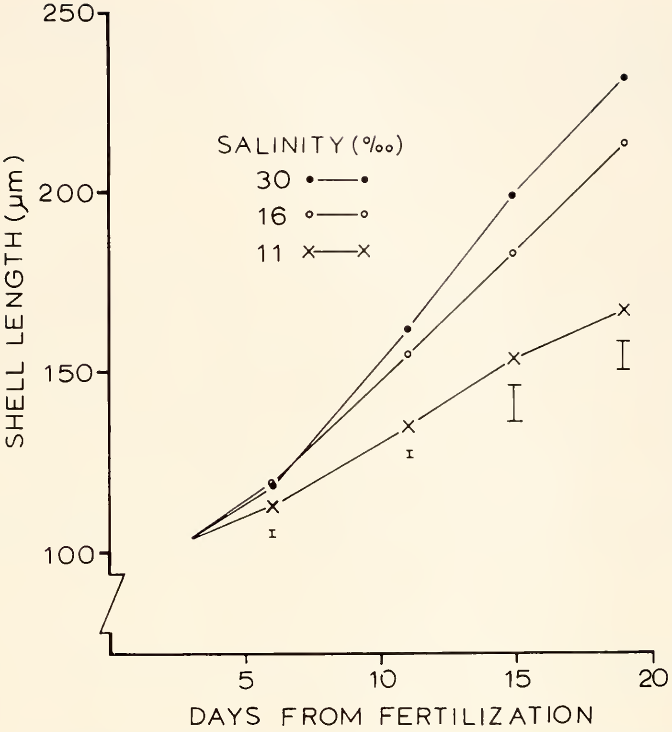

FIGURE 2. Increase in mean length of larvae raised at three salinities after development until day 3 at 30‰. Vertical bars indicate the least significant difference between means for each day ($\alpha = 0.05$).

effects and salinity as a fixed effect. In the single-pair experiment, random families and fixed salinities were used as main effects in a two-way mixed model design.

RESULTS

The effect of salinity

In the first salinity experiment no significant difference in mean larval length after 18 days of growth could be detected between larvae raised at 30‰ and those raised at 18‰. In the second experiment salinities lower than 18‰ produced a marked decrease in larval growth (Fig. 2). The first detectable response was noted three days after the salinity was dropped from 30‰ to 11‰. By day 11 a slower rate of growth was also detectable in the 16‰ treatment. Except for this slower growth, larvae in the two low salinity treatments showed no abnormal development, and swimming behavior was just as vigorous as in the 30‰ group. Although no counts were made, mortality appeared to be very low with no obvious difference among the three treatments.

These results differ from those of Brenko and Calabrese (1969), who found reduced growth at a salinity of 25‰. This may reflect genetic differences in larval response to salinity between mussel populations. Unpublished observations and the work of Bayne (1965) suggest that larval growth at different salinities can depend on the population from which the parents were derived.

No significant difference was found among replicated beakers in these experiments allowing for a single beaker to be used for each treatment in the genetic experiments.

The genetic experiments

From the factorial mating experiment at two salinities males, females, salinities and all interactions had a significant effect on 16 day larval length (Table I). A substantial proportion (23%) of this larval variation could be accounted for by genetic and interaction differences among families. This is actually an underestimate, since some of this variation remains inseparable from the 77% within-family variation. Examining the components of variation showed males contributed about twice as much to the total as females (5.4% compared to 2.4%). This may be a function of the small number of males and females used rather than any differences due to sex alone. The interaction between males and females was slightly larger, making up 6.3% of the total variation. Families responded differently to changes in salinity indicating the presence of genetic interactions with salinity.

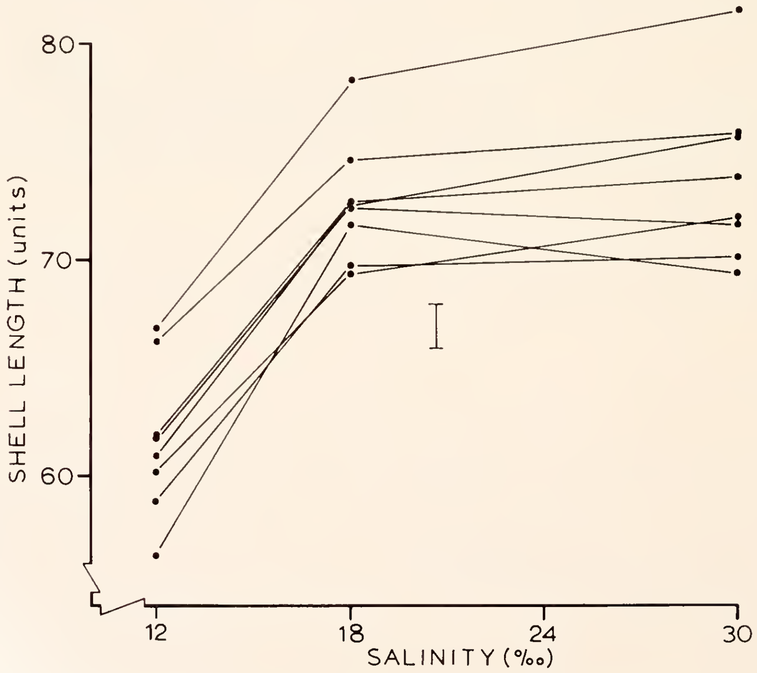

FIGURE 3. Mean length (1 unit = 2.74 μm) of 16 day old larvae from eight single-pair families raised at three salinities showing responses of families to salinities and interactions between families and salinities. Vertical bar indicates the least significant difference between means ($\alpha = 0.05$).

TABLE I

ANOVA *on 16 day larval length for factorial mating experiment raised at 30‰ and 12‰.*

Source of variation	df	Mean square	Component of variation (%)
Main effects			
Salinity (S)	1	99,322.8**	
Female (F)	5	268.5*	2.4
Male (M)	5	499.7**	5.4
2-way interactions			
S × F	5	165.0**	3.5
S × M	5	125.9**	2.5
F × M	25	91.9**	6.3
3-way interaction			
S × F × M	25	32.1**	3.2
Residual	2808	12.1	77.0
Total	2879		100.3

* $P < 0.05$.
** $P < 0.01$.

The salinity-female and salinity-male interactions were similar (3.5% and 2.5%, respectively) and seemed to be as important as the male and female components themselves. An appreciable (3.2%) component due to a salinity-male-female interaction was present.

The male and female effects and the male-female interaction effect are due to a genetic component of variation contributed by each individual parent in the experiment. Male and female variance components estimate 1/4 of the additive genetic variation, as well as any sex differences, while the male-female interaction estimates 1/4 of the dominance variation. The relative importance of additive genetic variance for a trait compared to the total phenotypic variation is a measure of its heritability in the population. Estimated heritabilities are used by quantitative geneticists to predict responses to selection. Such a detailed genetic analysis is beyond the scope of the present study, and a treatment of the subject can be found in Falconer (1960) and Becker (1975).

In the experiment involving eight single-pair families at three salinities, families, salinities and their interaction were all highly significant in influencing 16 day larval length (Table II). Variation among families represents a large portion of the genetic variation (1/2 additive and 1/4 dominance) and made up a substantial amount (27%) of the total observed variation in larval length. The interaction of this genetic component with salinity, although significant, was not as large (3% of the total). The remaining 70% could be attributed to variation within each family, comparable to the 77% of the previous experiment. Figure 3 plots family means at the three salinities, illustrating the sources of variation and their effect on larval length. The response to these salinities, averaged over families, showed no significant difference between the 18 and 30‰ salinity levels but a significant decrease

TABLE II

ANOVA *on 16 day larval length for single-pair families raised at 30‰, 18‰ and 12‰.*

Source of variation	df	Mean square	Component of variation (%)
Salinities (S)	2	14,478.1*	
Families (F)	7	1,288.1*	27
S × F	14	77.1*	3
Residual	936	27.8	70
Total	959		100

* $P < 0.01$.

in mean length at 12‰. This pattern agrees with the results found in the salinity experiments.

DISCUSSION

The salinity environment experienced by the Ostrea Lake mussel population during spawning is characterized by a low in the early spring, followed by a rise over the summer months (Fig. 1), a pattern that seems to be repeated each year (MacLeod, personal communication). Depending on when some adults spawned, larvae from this population may very well encounter salinities ranging from 18‰ to almost 30‰. Results of the experiments showed no average effect of these salinities on larval growth, suggesting that the population is prepared to cope with such a salinity range during the pelagic larval period. Salinities lower than this significantly decreased growth. A similar pattern of response was observed by Davis (1958) for larvae of *Venus mercenaria* and *Crassostrea virginica.*

There tends to be a general reduction in larval growth for bivalve species raised at reduced salinity (Davis, 1958; Davis and Ansell, 1962; Davis and Calabrese, 1964; Brenko and Calabrese, 1969; Calabrese, 1969; Lough and Gonor, 1971, 1973). In some cases this is accompanied by a large increase in mortality (Brenko and Calabrese, 1969; Calabrese, 1969), indicating that some sort of physiological stress may be hampering normal growth. In the present experiments with *Mytilus,* normal behavior was observed with no obvious mortality, even at the lowest salinity tested. Slow growth in this case could just be a nonfatal consequence of some physiological response mussel larvae have for surviving at reduced salinities. Little is known of larval physiology, making it difficult to speculate on a possible mechanism, although it may be related to energy requirements. Lough and Gonor (1973) measured oxygen consumption of *Adula californiensis* larvae exposed to different salinities, but could not detect any significant effect. This could possibly be due to poor culture conditions, since they observed very little growth over a sufficiently long period of time.

In a random sample of mussel larvae from a population, observed variation in a metric trait, such as larval length, is the result of genetic differences among individuals and differences in the microenvironment in which they have been growing. The two genetic experiments show that a substantial amount of this phenotypic variation is of genetic origin. This is consistent with the general conclusion from electrophoretic data that *Mytilus edulis* populations possess a large amount of genetic variability (Levinton and Koehn, 1976). Indeed, breeding studies themselves can be used as evidence for the existence of high levels of genetic variability in natural populations (Lewontin, 1974).

Families of larvae were raised at different salinities to determine if genetic response depends on salinity level. Significant interactions with salinity found in these experiments indicated the presence of genes, affecting larval growth, which depend on the level of the salinity environment for their expression (Falconer and Latyszewski, 1952; Scheinberg, 1973). That is, the relative importance of genes having an influence was different at the various salinities. This close connection between genes and salinity is not surprising, since salinity variations are very much a part of the estuarine habitat of *Mytilus*. In this fluctuating environment, the selection of genes which are sensitive to changes in salinity might be part of a mechanism for adjusting to different salinities (Speiss, 1968; Fontdevila, 1970). Laboratory studies on a number of organisms and traits have detected similar kinds of genotype-environment interaction (Speiss, 1968). Selection experiments on *Drosophila* at various temperatures indicated the presence of genes influencing wing length only at specific temperatures, as well as genes affecting wing length at all temperatures (Druger, 1962). The role of this additional source of genetic variation in populations and its relationship to changing environments is not clearly understood. Progress is being made using genetically defined laboratory populations, but little has been attempted with natural populations (Westerman and Lawrence, 1970; Westerman, 1971; Fontdevila, 1973).

Due to the small number of individuals represented in the experiments, it is difficult to attach any meaningful estimate to the Ostrea Lake population of the amount and kinds of genetic variability present. However, it must be reasonably high, as indicated by the highly significant results obtained. The importance of this genetic variability for larval growth appears to be almost equalled by its interaction with salinity. As pointed out by Westerman and Lawrence (1970), this interaction complicates a genetic analysis of the structure of a population under a single set of conditions. Its presence, however, brings up the question of its evolutionary significance. In the present study such interaction may in some way be related to past selective influences of the salinity environment on previous generations. Levins (1968) proposed that populations could be composed of a range of genotypes, each adapted to a specific environment through genotype-environment interaction, as an adaptation to a fluctuating environment. Without more extensive experiments, it is difficult to see an adaptive relationship between larval growth rate and salinity. Slower growth under certain salinity conditions, however, would extend the pelagic period (Bayne, 1965), increasing the probability of encountering a more "suitable" salinity environment for settlement. Salinity is, of course, only one of several important factors of concern to *Mytilus* larvae and adults (Scheltema, 1965).

With the increasing number of marine invertebrates which can be cultured through their complete life cycle, breeding studies are beginning to be used to obtain information on the genetic structure of various populations. This enables a valuable approach to understanding the relationship between genetic variability for specific traits of a species and the interaction with environmental parameters thought to be of some importance. It would be interesting to extend the results obtained here to other populations of *Mytilus edulis* experiencing contrasting salinity conditions.

We would like to thank G. F. Newkirk and R. W. Fairfull for valuable suggestions on the experimental and statistical techniques used in this study. R. W. Doyle and E. Zouros provided useful comments. L. MacLeod was kind enough to allow the use of the facilities at Ostrea Lake. The senior author was supported by a Dalhousie University Graduate Fellowship and the research was supported by a grant from the National Research Council of Canada.

SUMMARY

1. Adult specimens of *Mytilus edulis* in spawning condition were collected from a shallow, enclosed bay which was known to experience fluctuations in salinity. Growth of the resulting larvae was measured under different salinity conditions in the laboratory.

2. There was no significant difference in mean length between larvae raised at 30‰ and those raised at 18‰ after 18 days of growth. Salinities below this significantly decreased growth.

3. Families of larvae of known parentage were raised at different salinities.

4. The genetic analysis indicated substantial genetic variation in larval length measured 16 days after fertilization, as well as significant genetic interaction with salinity.

5. This is interpreted as the presence of genes influencing larval growth which depend on salinity for their expression and may be related to the past selective influence of a fluctuating environment.

6. These quantitative genetic techniques provide a useful approach to studying genetic variation in marine organisms and its interaction with the environment.

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MAINTENANCE AND MATURATION OF SQUID (*ILLEX ILLECEBROSUS*) IN A 15 METER CIRCULAR POOL

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Most interest in the laboratory maintenance of squid has centered on their use as a source of giant nerve fibers for neurophysiological studies. Although a number of smaller, partially benthic, cephalopods have been successfully reared (see Boletzky, 1974, and Gabe, 1976, for reviews), there is, as yet, no standard laboratory cephalopod; and long term maintenance of the larger teuthids with giant axons remains a problem. Summers, McMahon and Ruppert (1974), having evaluated a number of factors contributing to the mortality of *Loligo pealei* in captivity, conclude that skin damage resulting from contact with tank walls is a major factor in their limited survival (15 days maximum mean survival time) in small (1.68 m^2 area) rectangular tanks. Mikulich and Kozak (1971) and Matsumoto (1976) have reported slightly longer survival of *Todarodes pacificus* and *Doryteuthis bleekeri*, respectively, in larger (25 m^2 area) and circular (1.76 m^2 area) tanks. For *Illex illecebrosus* in captivity at the Marine Sciences Research Laboratory, Newfoundland, typical survival times of one to two weeks have been reported (Rowe and Mangold, 1975), but recently with larger tanks some animals have survived for 60 to 80 days (C. C. Lu, Memorial University of Newfoundland, personal communciation). While *I. illecebrosus* is not used for giant axon studies, it is similar in size and behavior to the species that are, and the results of this study suggest that, given a healthy starting stock and an adequate volume of sea water, long term maintenance of this species and probably others is possible.

The present experiments were undertaken to determine whether chronic studies on the endocrinology of *I. illecebrosus* were feasible using the 15 m diameter tank in the Aquatron Laboratory at Dalhousie University and to learn more about the species' reproductive biology. Information on the reproduction and life cycle of *I. illecebrosus* should be of value in rationalizing an increasing commercial fishery (Mercer, 1973a). This report deals primarily with survival and with techniques suited to chronic studies; however, maturation is also discussed since most cephalopods die shortly after spawning, and degenerative changes associated with maturation are likely to influence survival.

MATERIALS AND METHODS

Animal collection

Squid were obtained as a by-catch from a local mackerel fisherman and were caught in a floating box trap located on the south side of Herring Cove, near the mouth of Halifax Harbor in water of 16 m depth. The 35 mm mesh net box is "L" shaped with outer dimensions of 40 m and 24 m and is 12 m wide and deep at all points. The short leg of the "L" faces the shore and a 65 m "leader" of 115 mm mesh netting runs from the shore to a 3 m door, located at the inner angle, which can be closed after the catch enters. Squid were most frequently caught during spring tides. The wild population samples indicated in Figure 2A represent the majority, but not all of the seasons catches. Individual catches ranged from a few animals to several thousand, but small catches were most common in late May and early June when the squid are small enough to pass relatively easily through the "leader". The trap is certainly size-selective and this may account, in part, for the fact that over 90% of the squid were female since *I. illecebrosus* females are typically larger than males (Squire, 1967) ; however, the sex ratio changed little later in the season when males were much larger than early season females. The apparent steady growth during the season suggests that the squid belonged to a single modal class, as described by Squires (1967).

A total of 85 live *I. illecebrosus* was collected in three lots from the trap. For live collection, the floor and walls of the trap were raised to confine the squid to a small volume of water, and individual animals were transferred from the trap in buckets of water to 60 × 90 cm fiberglass tanks filled to a depth of 30 cm. A maximum of 20 squid were held in each tank aboard the tending vessel with a continuous, copious flow of water for periods of about one hour during the 25 km trip to a dock near the Aquatron. The tanks were then transferred to a truck and supplied with air from a battery driven compressor during the 10 to 15 minutes needed to move them to the Aquatron pool. The portable tanks were lowered into the isolation pool (Fig. 1), floated into the main pool, and submerged to release the squid with minimum handling.

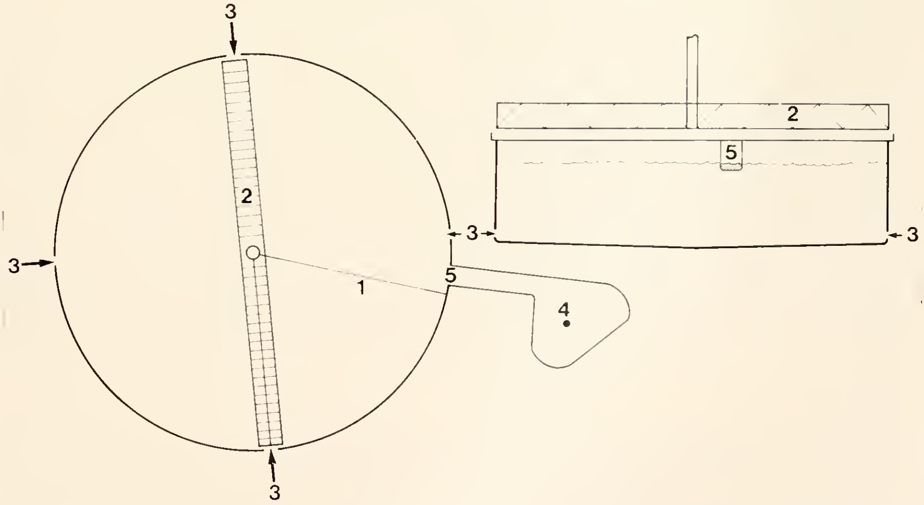

FIGURE 1. Plan and sectional views of the pool tank in the Aquatron Laboratory at Dalhousie University where squid were kept. Features discussed in the text are: 1) restricting curtain, 2) axial bridge, 3) water supply inlets, 4) drain in isolation pool, and 5) weir in connecting channel. Section is in the plane of the bridge.

Holding facility

The indoor circular pool tank in the Aquatron Laboratory is 15.24 m in diameter, and its sloping bottom ranges in depth from 3.55 to 3.91 m at the center (Fig. 1). It was constructed of reinforced concrete, lined with multiple, sprayed coats of light olive-colored polyvinylchloride (PVC). Twenty-two underwater windows, each approximately 1 m square, are located around the perimeter of the tank providing viewing at all depths. The entire tank rests on neoprene blocks providing partial acoustical uncoupling. The connected isolation pool is 1.12 m × 1.8 m diameter. For the present study, the water level was held 0.5 m below maximum to prevent the squid from jetting into the scuppers, giving a total water volume of 580 m^3. The 28 supply inlets, which were not in use, were blocked with 1 cm mesh PVC screening to prevent the squid from becoming trapped in the blind-ended pipes.

An axially suspended bridge spans the full width of the pool, and can be rotated mechanically from a remote control unit on the bridge to give access to any point on the surface. The pool is also equipped with a "vacuum cleaner" driven by hydrostatic pressure used to clean the bottom during long-term maintenance. For a more detailed description of the Aquatron Laboratory see O'Dor, Durward, Steadman and Balch, 1977.

Water supply

Sea water was pumped from the Northwest Arm of Halifax Harbor through intakes located at a depth of 15 m, 0.7 m off the bottom. Although water quality is relatively high since the Arm is well flushed and since effluent and storm drainage is normally restricted to the surface layer, the water was routinely passed through four Graver pressure sand filters which were backwashed daily. Water flow into the main pool was through the bottom inlets opposite the isolation pool at rates of 100 to 150 liters/minute (Fig. 1). Drainage of the main pool *via* the isolation pool was over a weir providing surface skimming to remove feces and the warmest surface water. Temperature varied throughout the pool by less than 1° C and was generally 2 to 3° C above the intake temperature (Fig. 2). Temperatures varied from 17° C in September to 7° C in December, approximately the optimal temperature range for *I. illecebrosus* reported by Squires (1957). Although water is warmed 1 to 2° C as it enters the Laboratory there was no evidence of supersaturation in the pool; and although no measurements were made, O_2 levels were presumably similar to those in the ocean, since the water was not recycled and the volume was very large for so few animals. Salinity during these experiments varied from 29.5 to 32.0‰, but were very close to 30.0‰ during most of the period.

Lighting

The pool is isolated from natural light except for three small windows above the water surface on one side which receive indirect skylight. This is a very small percentage of the artificial light provided by six 400 watt Sylvania Metalarc Lamps (400 BU-HOR) distributed uniformly over the surface at a height of 4 m. Light intensities in the lower 2 m of water where the squid usually stayed averaged 2 wm^{-2} and varied from 0.04 to 5.5 wm^{-2} in different areas. All of these values are within the normal range near bottom in the area of capture (Platt and Irwin, 1968). A constant, daily photoperiod of 15.5 hours, equivalent to the longest days during the experiments, was used throughout the experimental period to minimize the influence of natural light. The metal-halide lamps have a long warm-up period which eliminates sudden increases in light which frighten squid (LaRoe, 1971), and the on-off cycles of the individual lamps were staggered at 15 minute intervals to simulate dawn and dusk. Low levels of indirect light, approximating starlight, were provided through the small windows during the dark period.

Feeding

When squid were first placed in the pool, 100 to 200 live specimens of *Fundulus* spp., 5 to 10 cm in length, were also added. Most squid would feed on these fish within two hours of introduction. When the squid were accustomed to feeding in the pool, live specimens of *Fundulus* were tossed into a particular location one at a time until the squid came to feed. Once "trained" to this feeding area, they would take frozen smelt and chunks of frozen mackerel as they sank, although previously such food had been ignored. Food which fell to the bottom was never eaten, but could be easily cleaned from the small feeding area. Each squid received at least one 30 to 50 g chunk of mackerel per day, and certain individuals took two or three chunks before the others fed. Occasionally this diet was supplemented with live *Fundulus* which were preferred.

Handling

One major difficulty in using the large pool for chronic experiments was recapturing the squid. Initially, squid could easily be lured to the feeding area with a live *Fundulus* on a string and taken from the water with a 35 × 40 cm bait net, 15 cm deep, made from 5 mm knotless nylon netting to minimize skin damage; but after one or two experiences, a squid would learn to distinguish free-swimming *Fundulus* from those with strings attached and would no longer approach. As a more general but tedious solution to the capture problem, a curtain was placed across the pool such that half of the curtain hung fixed to a rope running from the side, near the entrance to the isolation pool, to a swivel at the center of the bridge, while the other half hung from the rotating bridge itself (Fig. 1). Thus the curtain could be folded back on itself so that it blocked only half the width of the pool, giving the squid access to the entire pool. Rotating the bridge slowly through 350° forced all of the squid into a small wedge, opening at its outer edge to the isolation pool. The squid could be captured in the restricted area of the wedge or herded with hand nets into the isolation pool. The curtain was made of 0.05 mm thick black polyethylene sheeting with 2 cm holes every 50 cm in a grid pattern to allow water to flow through. The holes were punched with a cork borer so that flaps remained attached at the bottom; and these flaps, floating up to close the holes, gave the stationary curtain the appearance of a continuous wall. It was fitted to the sloping pool bottom and weighted. A fine netting curtain would have moved more rapidly through the water, but the risk of entanglement and skin damage would have been greater.

Marking

A second difficulty with chronic experiments in a tank with large numbers of squid is identification, which was necessary when squid from two groups were in the pool at the same time and when individuals were measured and inspected more than once. Tags placed in any part of the body were either removed or resulted in self-mutilation, and fin notches tended to interfere with swimming and to become infected. The most effective marks were patterns of large dots tattooed onto the fins using India ink gravity fed through a 30 gauge needle on an open syringe. A few passes through the fin at a single location left a dot, which remained throughout the experiments, and was visible from above during capture or when viewing through underwater ports. Even when these dots occasionally became infected, they were still identifiable.

Tattooing was carried out under an anesthetic. After netting, the squid were transferred with wet hands to a 40 × 14 × 14 cm polyethylene container with a tightly fitting lid (a "bread saver") containing 3 to 4 liters of 3% urethane in sea water at approximately the same temperature as the pool. When respiratory movements stopped, after two to five minutes depending on temperature and animal size, the animals could be injected, tattooed or inspected by inserting a 2 cm diameter glass tube into the mantle opening. Animals were wrapped in wet towels during the procedures to prevent abrasions, drying and contact with dry surfaces. More complex surgical procedures of the sort previously carried out in *Octopus vulgaris* (O'Dor and Wells, 1973), including brain lesions and optic gland removal, could be carried out when the squid were left in the anesthetic until the "eye blink" reflex almost disappeared (usually three to four minutes after respiratory movements ceased). Recovery from light anesthesia occurred after the mantle cavity had been flushed with a gentle stream of sea water for four to five minutes, but recovery from the deeper anesthesia often took up to fifteen minutes.

RESULTS

Survival

Trapped squid were placed in the pool on three occasions in 1976: 15 squid on July 15 (group A), 35 squid on September 13 (group B), and 35 squid on October 24 (group C). Maximum survival times in each group, as indicated in Figure 2A, ranged from 32 days for group C to 82 days for group B. Although few group C animals were used for experiments, this group had the lowest mean survival time, 13 days. Realistic mean survival times cannot be given for groups A and B since the majority of the animals in these groups, including the longest lived, were killed for other experiments; however, minimum values calculated from deaths from all causes are 26 days and 35 days, respectively. Figure 3B illustrates how conservative these values are, since in group B only two of the deaths after day 50 were natural, and even these were remarkable. The second to last natural death was the only observed instance of cannibalism; the sole male in the group was found partially eaten. The last natural death was the first female to become fully mature. It had behaved unusually for several days prior to death, and 23% of its 442 g body weight was eggs in the oviducts ready to be laid.

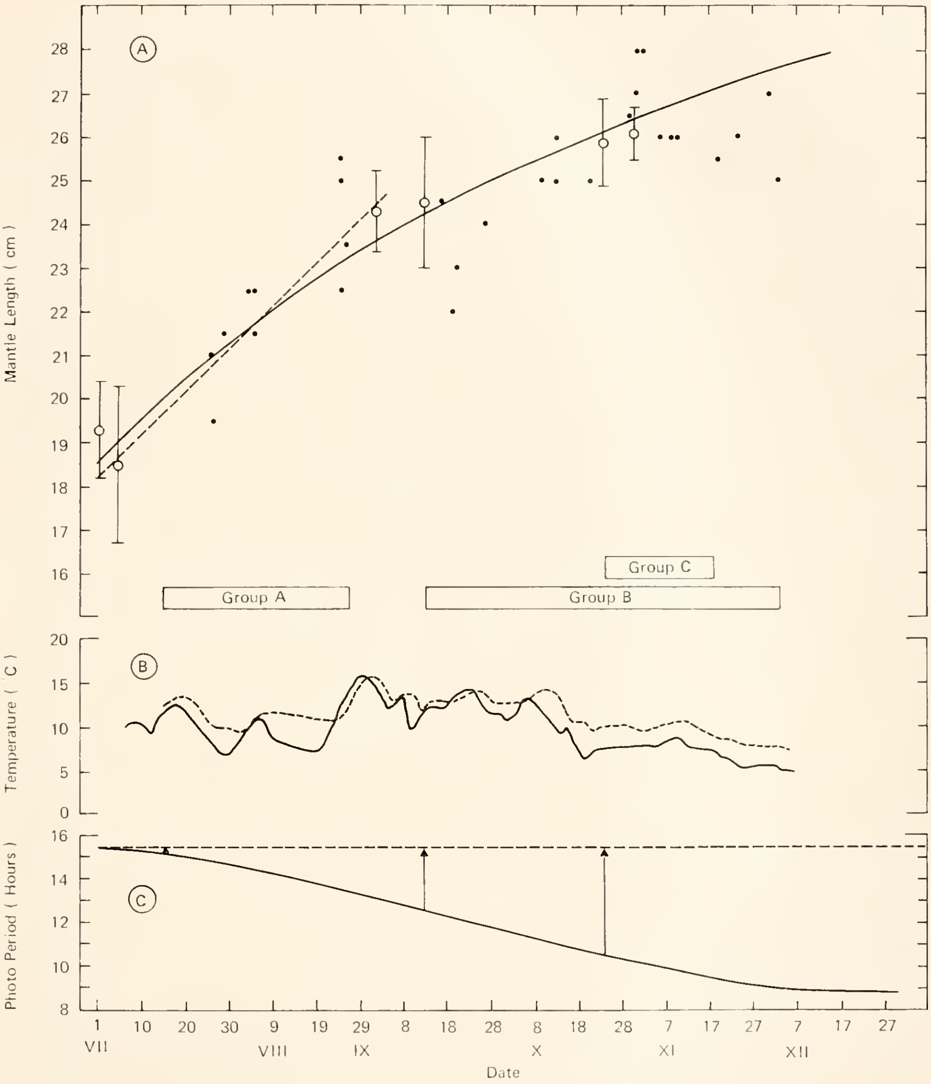

FIGURE 2. Physical conditions and growth of squid during the 1976 experiments. (A) Open circles and bars indicate mean mantle length (ML) and standard deviations for females taken from the wild. Solid circles indicate ML at death for animals from groups A and B which were kept in the pool during the indicated periods. The solid line is a growth curve for squid off Newfoundland redrawn from Squires (1967). The broken line is the regression of ML *versus* time for animals in group A. (B) The solid line is the water temperature at the intake and the broken line, that in the pool. (C) The solid line shows the natural photoperiod at 45°N latitude during the experiment (sunup to sundown), while the broken line indicates the photoperiod in the pool. The arrows show the change in photoperiod experienced by squid transferred from natural to experimental conditions.

Growth and maturation

The mean mantle lengths (ML) for samples of squid collected from the Herring Cove trap throughout the season, shown in Figure 2A, fit reasonably well on the growth curve given by Squires (1967) for *I. illecebrosus* off Newfoundland, suggesting that the population sampled in this study was fairly uniform, with a similar growth rate. Included in the figure are the mantle lengths at death of squid from groups A and B; these animals also appear to have grown at a similar rate. The linear regression of ML *vs.* time in the pool (broken line, Figure 2A) suggests a growth rate of 0.94 mm/day (correlation coefficient, 0.83) in group A, which is slightly greater than that reported by Squires (1967). However, the 15 squid in the pool were the total catch for that day so that no directly comparable data for ML at the start are available. A similar regression line can be drawn for group B for the first 50 days, but this line does not pass through the mean ML of the control group collected with B. In both groups early deaths of smaller squid appear to influence the regression so that calculated growth rates are slightly exaggerated; however, some growth is indicated in both groups. The average ML in group B was 1 cm larger at death than the average ML of controls killed on September 13. This was not a significant increase ($P < 0.1$ by Student's *t*-test); however, for the squid which were in the pool for more than 40 days, the mean ML is 2 cm greater which is highly significant ($P < 0.001$, Mann-Whitney U-test). This is partly attributable to deaths of small animals, as indicated by a decreased variance in the later group, but the probability of the largest animals in this group having been present in the population at the start of the experiment is only 0.01.

The fact that the group B animals began to mature sexually after 30 to 40 days probably reduced their overall somatic growth. Nidamental gland length (NGL) was used as an index of sexual maturity, and as Figure 3A shows, it increased nearly four-fold in the pool in 50 days, while in animals from the sea the increase was only about 25% over the entire season. Eight of the fourteen squid remaining on October 24 were anesthetized and marked, and their mean NGL as measured through the mantle (to the nearest 5 mm) was 70 ± 11 mm. The mean NGL at death over the next 40 days for this group was 104 ± 13. The average ovary weight (± s.d.) at death in this group was 72 ± 31 g and that for squid from the sea at the same time was 2.2 ± 0.7 g. Development of these large glands and ovaries was so extensive that they could easily be seen in the living squid from the observation windows. Somatic growth must have stopped as reproductive growth increased, since there was no further increase in ML among the marked squid; however, as they continued to feed, there were probably further increases in weight due to ovarian development. All of the animals eventually ceased to feed, even on live *Fundulus*, as the ovaries developed. We estimate from visual observations that ovaries weighed approximately 50 g when feeding stopped. Although no measurements were taken, the digestive system appeared to be greatly reduced in size when the fully developed animals were dissected.

Forty days in the pool produced a significant increase in NGL in group B, but squid from group A which survived for a similar period showed no increase. The four longest survivors in group C were killed for experiments after 20 to 30 days in the pool, when examination under anesthetic indicated that their ova were in

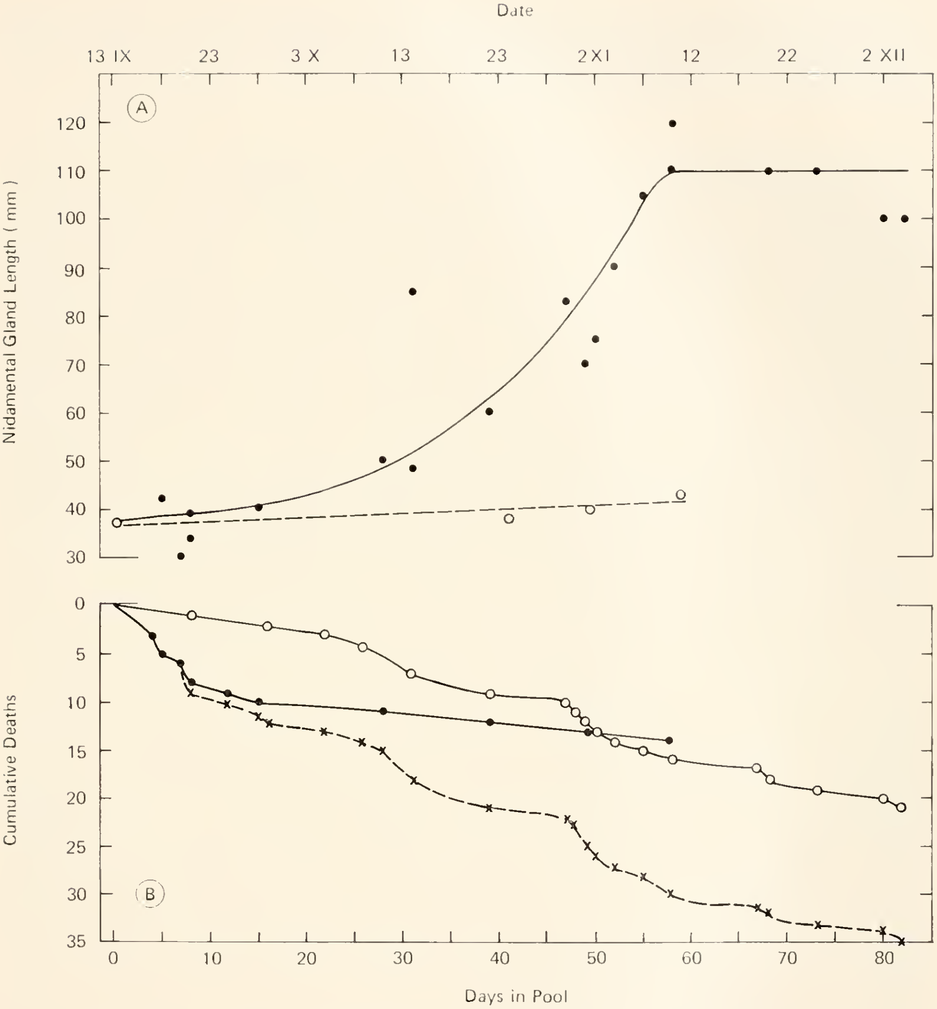

FIGURE 3. Deaths and nidamental gland lengths (NGL) at death for squid in group B. (A) Open circles are mean NGL for animals from the wild. Solid circles for NGL at death for group B animals in the pool. (B) The broken line is a record of total deaths in group B and is the sum of the natural deaths (solid circles) and animals killed for experiments (open circles).

the early stages of vitellogenesis. Average NGL in these animals was 58 mm, significantly ($P < 0.01$ by t-test) larger than in comparable animals from the sea.

Survival after surgery

There were no deaths attributable to light anesthesia with urethane, tattooing or inspection; however, recovery from deep anesthesia was much less sure, particularly if the surgery required more than about five minutes. The recovery rate was about

50% in this latter group; animals which had had optic glands removed were killed after two days for other experiments, but the only animal to recover after removal of the vertical lobe of the brain survived for seven days.

DISCUSSION

The observations reported here agree with the suggestion (Summers *et al.*, 1974; LaRoe, 1971) that the major cause of early mortality in captive squid is skin damage. Squid have very delicate skin and apparently little capacity for healing when lesions are large. Squid which came into the pool with lesioned areas amounting to more than 2 to 3 cm^2 over their body surface never survived for more than a few days. However, individuals of *I. illecebrosus* and probably other species of squid can be maintained if they are brought to the laboratory without skin damage and are kept in conditions which minimize subsequent damage. It seems likely that the majority of the animals in groups A and B would have lived out their normal life spans had not the experimenters or prococious maturation interferred. When group C, which had the shortest mean survival time, was taken, the trap contained 5000 to 6000 squid, a much higher density than the other groups. Within 24 hours nearly all of the animals showed many skin lesions. The four that survived for more than ten days were essentially unmarked, fed well and were in excellent condition when killed. In previous years when specimens of *I. illecebrosus* and *Loligo pealei* (from predominately male schools of *L. pealei* present near Halifax in May and June before the major influx of *I. illecebrosus*) were collected for the pool by trawling, their condition and mean survival times were usually about the same as group C's; thus, results were similar to those reported by Summers *et al.* (1974). Survival times of trapped squid kept in the Aquatron tower tank (10.5 × 3.7 m diameter) were also low, probably as a result of large skin lesions which appeared after frequent bumping and circling of the tank with the point of the mantle and fin edges rubbing the walls. When frightened, squid jet backward, and in small tanks they hit the walls, which seems to frighten them further and leads to continued jetting. In small circular tanks they circle, abrading a small area on one fin, while in rectangular tanks they move along one side until they reach a corner where they bump for long periods, often abrading large areas on their posterior surfaces (Summers *et al.*, 1974). Of the two behaviors, circling probably damages a smaller skin area and seems the easier pattern to break. On this basis circular tanks seem slightly preferable.

Summers and McMahon (1974) have presented an elegant analysis of appropriate tank dimensions for *L. pealei* of various sizes, based on the concept of "squid mean free path". In the large pool, even when frightened, the squid rarely contacted the walls and generally remained in a fairly compact school slowly circling the pool or drifting back and forth one or two meters in from the wall. When an observer first appeared at the edge or on the bridge, the squid usually moved to the far side of the pool but would return if the observer made no further movements. After a period without food the school would actually approach a visitor, apparently in hopes of being fed. The response to an observer at a view port was similar, but on one occasion, when a large group of school children were observing through all of the view ports simultaneously, the squid withdrew to the center of the pool and became quite agitated. When the vacuum cleaner or nets were used to remove debris from the pool bottom the squid would move away to maintain a reasonable distance, but were not greatly disturbed by the operation. The general impression was that the squid preferred to keep a certain minimum distance between themselves and any potential danger. This distance may also be a factor in determining the minimum size of tanks in which squid can "relax". From our results we can make the empirical observation that for *I. illecebrosus* of about 20 cm ML, a 3.7 m diameter is too small and a 15 m diameter is large enough.

If a 15 m tank is essential it should, at least, be possible to keep large numbers of squid in it. Summers and McMahon (1974) found no evidence of detrimental effects from crowding at densities of up to 16 squid/m^3. This density would be equivalent to 9000 squid in the pool. The maximum number we have held at one time was 50 when group C was added to the remainder of group B. This addition had no ill effects on group B, and there was no evidence that any of the problems of group C were a result of interactions with group B. The squid from both groups immediately formed a compact school and those from group C fed normally on *Fundulus* until they became moribund. While feeding and cleaning up after 9000 squid is beyond contemplation, the fact that they remain in compact schools suggests that several hundred would not be an unreasonable number in a large pool. A good strategy for keeping many squid for long periods would be to construct a single circular tank as close to 15 m in diameter as was economically feasible and to fill it with trapped squid as they became available. Trapping is not as reliable a means of catching squid as trawling (Summers and McMahon, 1970), but the longer survival of trapped squid should more than compensate for the difference.

Aside from skin damage, the major factor limiting survival in squid appears to be sexual maturation, which in most studied cephalopods is associated with degeneration of organs and tissues not related to reproduction, cessation of feeding and ultimately with death shortly after spawning (Van Heukelem, 1973). This was not expected to be a problem, since about 95% of the animals collected were female and in several years of collecting locally throughout the season a maturing female *I. illecebrosus* had never been seen. This is not just a local phenomenon as there are few records in the literature of mature females, and it is thought that they move out into deeper waters before maturing (Squires, 1957; Mercer, 1973b). In fact, the use of surgical techniques previously applied to *Octopus vulgaris* to induce maturation was planned (O'Dor and Wells, 1973). This proved unnecessary since some factor in the pool environment caused rapid maturation of all animals in groups B and C surviving for more than about three weeks, although no maturation occurred in group A or in the wild population.

The only factor which has been specifically suggested to induce maturation in *I. illecebrosus* is starvation; Rowe and Mangold (1975) have shown that ovary and nidamental gland weights of unfed squid kept in the laboratory are significantly greater than those in wild populations within a few days, particularly in large animals (ML > 20 cm). They were, however, unable to feed their animals a diet adequate to prevent this reproductive development in the laboratory, so the question of what constitutes starvation is open. LaRoe (1971) suggests that loliginids should get 30 to 60% of their body weight in food daily and that they starve if the level is lower than 10–15%. Our squid received as much food as they would take in one daily feeding, a minimum of 17% of body weight in group A which grew at an apparently normal rate without maturing. The minimum diet received by groups B and C was only 12.5% of body weight, since they were larger, but several squid (apparently the dominant members of the school; Arnold, 1962) consistently took up to three times this minimum and clearly grew. As all group B animals matured, starvation seems unlikely to be the common stimulus. While partial starvation cannot be ruled out as a contributing factor, some other feature of the laboratory environment, unrelated to food, must also have been involved in inducing maturation in our squid and possibly also in those of Rowe and Mangold (1975).

A second factor which might have influenced the maturation rate is temperature. The pool temperature was always a few degrees higher than that at 15 m in the Arm (Fig. 2), but how it compared with that experienced by the wild population is not known, since the vertical distribution of *I. illecebrosus* is not known with certainty. Most catch statistics for *I. illecebrosus* are based on incidental catches in ground fish trawls and prove only that the squid are present near the bottom in significant numbers during the day (Squires, 1957). Although there are no midwater trawl data to confirm it, photographic records of vertical distribution of the squid kept in the 10 m tower tank indicate that *I. illecebrosus,* like many other squid (Clarke and Lu, 1975), make a diurnal migration to the surface at night. The average depth during the night, 1.6 m, was significantly ($P < 0.01$) less than that during the day, 4.8 m (O'Dor *et al.,* 1977). Such diurnal migrations would expose the wild population in the area to temperatures varying from 2 to 5° C near the bottom to surface temperatures of up to 18° C (Platt and Irwin, 1968), but the average temperatures exprienced by squid in the wild are unlikely to have been higher than those in the pool. Richard (1966, 1970) has shown that in *Sepia* higher temperatures give increased somatic and gonadal growth, a result different from that in the pool where there was a dramatic acceleration of gonadal development while somatic growth continued normally or declined. The temperature effect in *Sepia* seems to result from a generalized increase in metabolism rather than a selective activation of the gonads *via* the optic glands which normally control sexual maturation in cephalopods (Wells and Wells, 1959). The optic glands in our mature specimens were greatly enlarged and orange (characteristics of secretory activity), and the rate of gonadal development was comparable with that seen in *Octopus vulgaris* after the inhibitory nerves to the optic glands were sectioned (Wells and Wells, 1959). The pool environment seems to have "triggered" a rapid maturation phase rather than merely to have accelerated an on-going process. In any case, the pool temperatures were dropping just at the time of greatest development in groups B and C and were relatively high for group A which showed no maturation. The wild population which also experienced dropping temperatures at this time, did not mature, so that temperature cues cannot explain our observations.

The factor which has been most consistently linked to activation of the optic glands and maturation in cephalopods is light, usually as it relates to photoperiod (Laubier-Bonichon and Mangold, 1975; Richard, 1967; Wells and Wells, 1959). When Richard exposed six month old female *Sepia* to light regimes ranging from 1 hour light in 24 hours to 23 hours light in 24 hours there was no response for six months, but at the end of this refractory period all animals receiving 12 or fewer hours of light matured within three months, while those receiving more than 12 hours of light matured at slower rates, apparently dependent on the photoperiod. We attempted to eliminate this variable by maintaining constant light intensity and photoperiod throughout the experiments; but in retrospect this may have been the wrong strategy, for while laboratory conditions remained constant those in nature did not. Group A was captured when the natural photoperiod was 15.1 hours and approximately this photoperiod was maintained in the pool. Group B's photoperiod changed from 12.5 hours to 15.5 hours on entering the laboratory and group C's from 10.5 hours to 15.5 hours. Thus, the two groups which matured received relatively large increases in photoperiod under experimental conditions. In comparing our results to those of Richard (1967), two interpretations are possible: first, Group A animals were refractory, but groups B and C were responding to the long photoperiod; or secondly, Groups B and C responded to the *change* in photoperiod, to which group A was not exposed. Both types of photoperiodic control are known to occur in other animals (Bünning, 1973).

It should be possible to determine which type of cue is the more likely natural one by looking at the life cycle of *I. illecebrosus.* Although the life cycle is not known completely, that part which is known appears to be quite regular. Squid of about 14 cm ML arrive on the Grand Banks and the Nova Scotian Shelf in late May, grow to about 28 cm ML and depart in early November (Squires, 1967 and personal observations). Females show virtually no sexual maturation during this period, but males may become fully developed. Squires (1967) has proposed a one year life cycle, based on rapid but not unreasonable growth rates, which requires that the young squid hatch in February. This cycle would be possible only if females could mate, spawn, and hatch their eggs in about 90 days between November and February. Females reached full maturity after as few as 55 days in the pool, so that if embryonic development requires less than 35 days, the cycle is feasible. There are no data on embryonic development of *I. illecebrosus,* but eggs of the closely related *I. coindetii* are the same size (1.0 × 0.8 mm) and develop in about six days at 20° C (Boletzky, Rowe and Aroles, 1973). Development times for cephalopod eggs are highly dependent on temperature as well as size, and Boletzky (1974) has shown for several species that with each 5° C decrease in temperature the time to hatching almost doubles. Although the eggs of *I. coindetii* from the Mediterranean did not develop at temperatures below 15° C (Boletzky *et al.*, 1973), extrapolation to 5° C, a temperature likely to be encountered in winter in bottom waters off the Grand Banks and Nova Scotian Shelf where *I. illecebrosus* presumably spawns, gives a time to hatching of 40 days. Development time for the eggs of *I. illecebrosus*, a species adapted to cold water (Roper, Lu and Mangold, 1969), is unlikely to be longer than this. If *I. illecebrosus* migrates to warmer waters to spawn, the time spent in migrating should be approximately balanced by decreased development time, supporting the proposed one year life cycle.

A simple assumption which would relate this natural cycle to photoperiodic stimulation of maturation in the pool is that migration and maturation are normally triggered in early November by the same cue. There is no actual increase in natural photoperiod until late December, but, as indicated in Figure 2C, the rate at which photoperiod decreases is reduced in early November at about the time migration begins. Rate of change of photoperiod is a cue known to affect sexual maturation in birds (Morris and Fox, 1958). If squid have a similar system, it would explain the natural cycle, and an absolute increase in photoperiod, as occurred in the pool, would likely trigger any system sensitive to more subtle rate changes. This is the only obvious cue which can explain all of the observations. If the smaller animals of group A were refractory, light intensity would remain a possibility; but for animals which move freely vertically and are exposed to a wide range of light intensities, it seems an unlikely cue. In any case, the tank offered a choice of intensities in the natural range.

On this basis, the best strategy for preventing maturation in *I. illecebrosus* and reducing the resultant mortality would be the use of a constant photoperiod equal to that on the shortest day of the year, since the naturally occurring decreases in photoperiod do not induce maturation in the wild. Maintenance in continuous darkness or low levels of light is a second alternative, but the effects of such unnatural regimes on maturation is difficult to predict (Laubier-Bonichon and Mangold, 1975). If precocious maturation can be eliminated, long-term maintenance and chronic experiments with *I. illecebrosus* and possibly other squid are feasible, given a healthy starting stock and a large pool.

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SUMMARY

1. Female specimens of *Illex illecebrosus* of 20 to 28 cm mantle lengths lived, fed and grew in a 15 m diameter pool for periods up to 82 days; deaths during the first week were associated with skin damage during capture and those after the eighth week with precocious sexual maturation.

2. Under the conditions described, squid survived repeated capture and urethane anesthesia, as well as tattooing and surgical procedures. Techniques for handling are described.

3. Although the cause of early maturation is not certain, squid entering the pool were exposed to increased photoperiods, and a relationship is hypothesized between this stimulus and the natural November spawning migration.

4. The rapid sexual maturation observed and the small size of eggs ready to be spawned indicates that the one year life cycle previously proposed for *Illex illecebrosus* is feasible.

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JUVENILE HORMONE AND VITELLOGENIN SYNTHESIS IN THE CECROPIA SILKWORM

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In many insect representatives from a broad spectrum of orders, vitellogenesis is controlled by juvenile hormone secreted by the corpora allata (see reviews by Telfer, 1965; Engelmann, 1970). Notable exceptions to this finding have been the saturniid moths (Lepidoptera: Saturniidae), in which the removal of the corpora allata from the pupa has no detectable effect on the production of eggs several weeks later (see Williams, 1952).

As first demonstrated by Telfer (1965), yolk formation in insects entails the sequestering of one or more sex-limited hemolymph proteins, the vitellogenins (Pan, Bell and Telfer, 1969). The synthesis of vitellogenins in the fat body in many non-saturniid insects requires juvenile hormone (see reviews by Wyatt, 1972; Doane, 1973). This observation raises the possibility that the failure of allatectomy to affect egg formation in saturniids may be related to an unusual timing sequence for vitellogenin production. In most insects, vitellogenin first appears in the blood of the adult at about the time that yolk deposition begins. However, most saturniids store a substantial quantity of this protein in the blood of the pupae well before the ovaries are able to use it (Telfer, 1954). Although the experiments of Williams (1952) with Cecropia clearly indicated that the ovarian components of yolk production are emancipated from juvenile hormone control, the possibility remained that the synthesis of vitellogenin still requires this hormone.

In this report, the effects of allatectomy of the early fourth and fifth larval instar of *Hyalophora cecropia* silkworm moths on the initial appearance of vitellogenin during larval-pupal molt and on the incorporation of amino acids into this protein during the pupal-adult molt, as well as the possible stimulation of vitellogenin synthesis in isolated pupal abdomen by a synthetic juvenile hormone, are described.

Materials and Methods

Experimental animals

Silkworms, *Hyalophora cecropia,* were raised on wild black cherry (*Prunus* sp.) in the field and sexed and staged according to Telfer (1967). Diapausing pupae were maintained at 5° C, and adult development was initiated by placing the pupae at 25° C. Pharate adults were also staged according to Telfer (1967).

Corpora allata and corpora cardiaca complexes were excised surgically (referred to as allatectomy hereafter; see Williams, 1961) from early fourth and fifth instar female larvae within three days after molting as described by Williams (1961). Following surgery, a small piece of Gelfoam (Upjohn) was put over the wound,

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and the caterpillars were placed at 6° C for about six hours and then returned to a netted cherry tree in the field. They were collected after cocoon construction and were examined and killed at the sign of tanning of the abdomen. Diapausing female pupae were allatectomized according to Williams (1961). The wound was sealed with a plastic cover glass. Control animals were similarly treated surgically, except the endocrine glands were not excised. Isolated abdomens were prepared from diapausing pupae by the method of Williams (1947), except that the midgut which is not involved in vitellogenin synthesis was excised. A tiny hole, for injection purposes, sealed with wax was made on the plastic cover glass used to cover the wound.

Immunochemical methods

The preparation of antibodies against vitellogenin, and their use in precipitating labeled vitellogenin from blood were described previously (Pan, 1971).

Oudin's single immunodiffusion test was used primarily for the detection of vitellogenin and was done according to Pan (1971). Ouchterlony double immunodiffusion was performed on microscope slides employing 0.8% agarose (Bio-Rad) dissolved in 0.15 M NaCl buffered with phosphates (pH 7.2). The wells were cut with a #13 gauge hollow needle. The distance between center and peripheral wells was 5 mm. Staining and recording of the precipitin bands are reported elsewhere (Pan and Wyatt, 1971).

Acrylamide gel electrophoresis

Disc acrylamide gel electrophoresis was done according to Davis (1964), with Bio-Rad model 150A electrophoresis cells, except 4% gel was employed and spacer gel was omitted. Sample was suspended in 40% sucrose made in running buffer. One per cent fast green FCF in 7% acetic acid was used to stain the protein bands. The vitellogenin band was determined by placing unfixed gels on specific antibodies mixed in 0.8% agarose and the position of the precipitin band was compared with bands in stained gels.

Labelling of vitellogenin in vivo

Except as noted, experimental animals were injected with 0.1 mCi ^{3}H-leucine (specific activity 5 Ci/mM, New England Nuclear Corp.) in 0.1 ml distilled water, with a few crystals of phenylthiourea added to prevent melanin formation in the animal. Following treatment, animals were held at 25° C and bled 12 hours after the injection. The preparation of blood for the determination of vitellogenin label and total blood protein label is reported in Pan (1971).

RESULTS

Synthesis of vitellogenin in allatectomized pharate adults

It has been reported that, during late pharate adult development of *H. cecropia*, more than half of the total blood protein radioactivity, following a single injection of tritiated leucine, was found to be in vitellogenin (Pan, 1971). Thus, this stage should be ideal for studies on the effect of the corpora allata on the synthesis of vitellogenin. The corpora allata-corpora cardiaca complexes were therefore removed from several pre-chilled diapausing female pupae. Operated pupae were allowed to initiate pharate adult development at 25° C. On day 18 of pharate adult development, when vitellogenin synthesis is at its peak (Pan, 1971), experimental and control animals were each injected with ^{3}H-leucine. Blood was collected 12 hours later and processed to determine the vitellogenin label and total blood protein label (see Materials and Methods). The results, shown in Table I, indicate that removal of the corpora allata in the pupal stage did not affect the incorporation of leucine into vitellogenin ($P > 0.5$) or total blood protein ($P > 0.2$) in late pharate adult development. When the vitellogenin label is expressed as a percentage of the total protein label (V/T% in Table I), no difference was observed between the experimental and control ($P > 0.2$). The expression of proportional incorporation as a percentage of total incorporation is a more meaningful method of relating specific blood protein synthesis, as has been discussed elsewhere (Pan and Wyatt, 1976).

Appearance of vitellogenin in pupae allatectomized as larvae

Although the results shown in the previous section clearly demonstrate that corpora allata are not required for the synthesis of vitellogenin in pharate adult Cecropia, the apparent normal synthesis of vitellogenin in these animals could be explained as the result of persistence of long-lived vitellogenin messengers before and after the surgery, in that surgical manipulation was performed months after synthesis of vitellogenin had been initiated (see Pan, 1971). Therefore, the possibility exists that the corpora allata are still necessary for the initiation of vitellogenin synthesis in Cecropia, as in many nonsaturniid insects. Corpora allata-corpora cardiaca complexes were then surgically removed from several dozen early fourth and fifth instar female larvae within three days after molting at a time when vitellogenin is not yet present in the blood, nor would it normally appear for at least one to two weeks after the surgery (Telfer, 1954). The presence of vitellogenin in these allatectomized individuals was then determined shortly before pupation, at which time the concentration of vitellogenin in blood is at maximum in normal animals (Telfer, 1954). The success of the surgery was indicated by the fact that those animals allatectomized at early fourth instar skipped the fifth instar stage entirely and molted into either miniature pupae or miniature pupal-adult

TABLE I

^{3}H-leucine incorporation into vitellogenin of pharate Cecropia adults allatectomized as pupae.

Animal	dpm × 10^3/5 μl blood*		V/T %
	Vitellogenin	Total blood protein	
Allatectomized	32 ± 4	53 ± 5	60 ± 2
Control	29 ± 2	46 ± 3	63 ± 1

* Mean ± s.e. (N = 4).

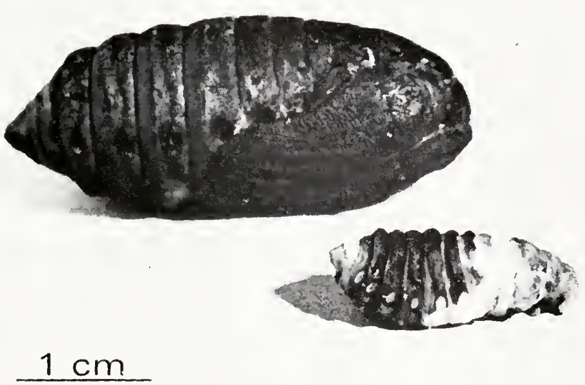

FIGURE 1. A miniature Cecropia female pupal-adult intermediate transformed from an allatectomized early fourth instar caterpillar. A normal pupa is in the background.

intermediates (Fig. 1); and those allatectomized at early fifth instar molted into pupal-adult intermediates (Fig. 2) essentially as described by Williams (1961). Morphological descriptions and cuticular protein analyses of these intermediates will be reported elsewhere (Willis and Pan, in preparation). Vitellogenin was detectable in all of the miniature pupae as well as the intermediates, indicating that allatectomy at an earlier stage did not prevent the future appearance of vitellogenin. It should be noted that the metamorphosis of these intermediates was so severely disturbed that they could not resorb their molting fluid and consequently could not molt; their old cuticle had to be removed manually. They also contained abnormally low volumes of blood and in several instances blood samples could not be obtained at all. In the later cases, several drops of saline solution were then added to the hemocoel, the washing was collected and the presence of vitellogenin was determined. For this reason, no quantitative analysis was performed on vitellogenin, only its presence was noted by immunodiffusion and several samples were analyzed also by acrylamide gel electrophoresis. Representative samples are shown in Figures 3 and 4.

Juvenile hormone and vitellogenin synthesis in isolated pupal abdomen

Blumenfeld and Schneiderman (1968) observed that, when diapausing pupae of *Antheraea polyphemus* (a saturniid) received a dose of the juvenile hormone extract (from male Cecropia adults) and transformed into "second pupae", the concentration of vitellogenin in the blood increased. The effect could be observed as early as 24 hours after hormone treatment. These authors concluded that juvenile hormone affected neither synthesis nor release of vitellogenin, but rather blocked its accumulation by the oocytes. It should be noted that it took 8–10 days for the diapausing animal to become a "second" pupa, whereas normal pupal-adult development requires 23 days. According to Telfer and Rutberg (1960), vitellogenin is not taken up by the oocytes before the twelfth day of pharate adult development. Since ovarian development was not observed in those "second pupae", increases in the concentration of vitellogenin in hemolymph of the "second pupae" of *A. polyphemus* could be related to a situation other than one in which the ovaries were unable to remove vitellogenin from the blood. Whether this increase in vitellogenin concentration resulted from juvenile hormone within the extract, or was in response to other factors present in the extract, remained unclear.

The possibility that juvenile hormone can promote the synthesis of vitellogenin, despite the fact that in the absence of the same hormones, vitellogenin continues to be synthesized, was tested on groups of isolated abdomens with a synthetic juvenile hormone (*dl*-JH). The advantage of using isolated abdomens over intact pupae is that the brain, ecdysial glands, corpora cardiaca and corpora allata are all excluded and any observed stimulation could then be attributed to the injected compound alone.

Groups of isolated Cecropia abdomens were prepared as described in Materials and Methods and kept at 25° C for at least two months before use, when the enhanced blood protein synthesis due to injury finally returned to the normal diapausing level (Telfer and Williams, 1960). Ten μg of Röller's JH-I in olive oil

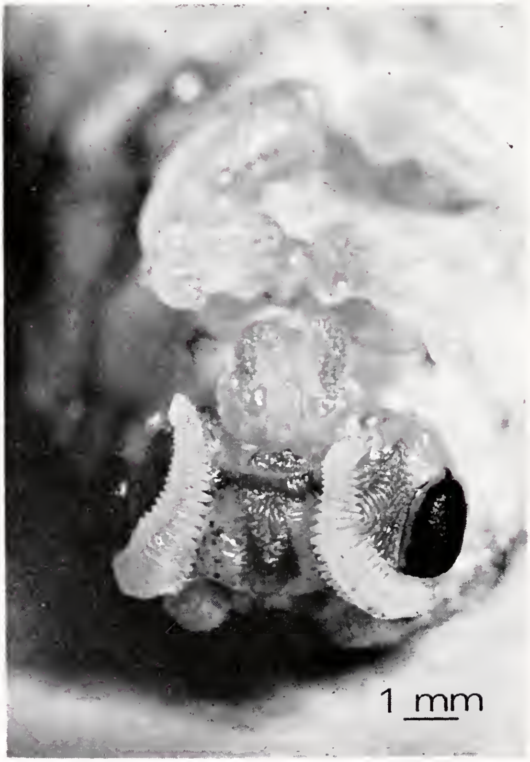

FIGURE 2. Close-up of a Cecropia female pupal-adult intermediate obtained by allatectomizing an early fifth instar caterpillar. Notice the pigmented compound eyes and barbed antennae.

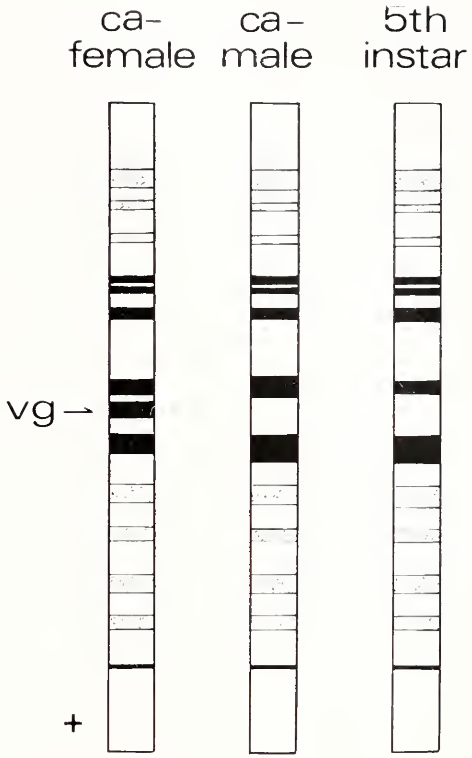

FIGURE 3. Acrylamide disc gel electrophoresis (4% gel) of the blood from a miniature Cecropia female pupa (left), a miniature male pupal-adult intermediate (middle) (both from allatectomized early fourth instar larvae) and a normal female fifth instar caterpillar (right). Vitellogenin (vg, arrow) is present only in the blood of the female miniature pupa; ca- indicates allatectomy. Cathode is at top of the gels. Stippled bands represent faintly stained protein bands. Leading band is the tracking dye whose position was also marked with injected India ink before staining.

(1 $\mu g/\mu l$) (a gift of Dr. G. R. Wyatt, Queen's University, Kingston, Ontario), a dosage sufficient to promote "second pupa" formation with Cecropia pupae (Judith Willis, University of Illinois, personal communication) were injected into each abdomen. Control abdomens received 10 μl of olive oil. Hormone and olive oil treatment lasted for 12, 24, 36 and 72 hours in respective groups. Six hours before each abdomen was opened for blood sample collection, 20 μCi of ^3H-leucine was administered to label the vitellogenin and other proteins. The results are given in Table II.

Based on incorporation into vitellogenin, measured as counts-per-minute (cpm), differences between JH-treated and control groups are not significant ($P > 0.3$), except 72 hours, where the cpm into vitellogenin exceeeded that into the controls ($P > 0.05$). However, the significance of this 72 hour difference is questionable, since differences between 12 and 72 hour JH-treated animals are not significant ($P > 0.1$). Differences are also not observed ($P > 0.4$) at either 24 or 48 hours,

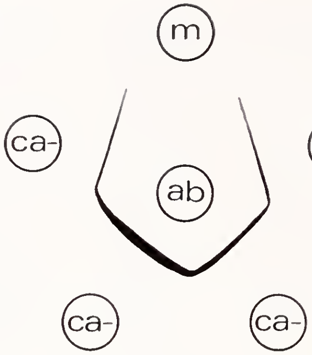

FIGURE 4. Ouchterlony's double immunodiffusion plate showing the presence of vitellogenin in the blood of three Cecropia female pupal-adult intermediates (ca-) transformed from allatectomized early fifth caterpillars, and a normal female diapausing pupa (f). Vitellogenin is absent from the blood of a normal male diapausing pupa (m). The center well (ab) contained antiserum specific for vitellogenin.

as compared to the 12 hour JH-treated group. When comparisons were made between the respective controls, no differences were noted ($P > 0.5$). Thus, incorporation into vitellogenin does not increase over a 72 hours period following JH administration into isolated abdomens.

A more meaningful method of analyzing vitellogenin incorporation is to normalize any stage-specific differences by expressing vitellogenin incorporation as a percentage of the total label incorporated (V/T% in Table II). This type of analysis also failed to uncover differences in vitellogenin incorporation following 72 hours of JH treatment ($P > 0.5$). Therefore, JH does not appear to stimulate the synthesis of vitellogenin in isolated Cecropia abdomens.

The general trend in increase in total blood protein incorporation in both JH-treated and control groups over a period of 72 hours can be attributed to the injury response (see Pan, 1971). Since cpm differences between JH-treated animals and controls of respective time points are not observed ($P > 0.5$), JH does not seem to

TABLE II

^{3}H-leucine incorporation into vitellogenin of isolated Cecropia abdomens treated with juvenile hormone.

Hours after JH treatment		cpm/5 μl blood*		V/T %
		Vitellogenin	Total blood protein	
12	JH-treated	21 ± 13	225 ± 93	7.5 ± 2.6
	Control	28 ± 23	232 ± 158	8.9 ± 2.4
24	JH-treated	33 ± 9	512 ± 327	8.9 ± 2.5
	Control	24 ± 5	296 ± 144	10.8 ± 2.8
48	JH-treated	32 ± 3	421 ± 132	9.5 ± 3.0
	Control	31 ± 15	493 ± 104	5.7 ± 1.8
72	JH-treated	48 ± 5	672 ± 32	7.1 ± 0.6
	Control	27 ± 3	539 ± 193	6.2 ± 2.2

* Mean ± s.e. (N = 3).

stimulate the synthesis of other blood proteins as a whole as well, although analysis of the individual protein component is necessary to clear up this point.

Experiments were terminated following 72 hours of treatment because preliminary experiments employing JH extract from adult male Cecropia silkmoths (see Williams, 1956; Gilbert and Schneiderman, 1960) failed to cause stimulation of vitellogenin synthesis over a two to fourteen day period (Pan, unpublished).

Discussion

Three lines of complementary evidence are presented in this report, indicating that the corpora allata and juvenile hormone are not required for the initial appearance and synthesis of vitellogenin in the Cecropia silkworm.

Wigglesworth (1964), in discussing why a hormonal stimulus should be needed by most insects for the full activity of the reproductive cycle, suggests that because egg production in most insects, like molting, is a cyclical process; it is desirable for the insects to have some mechanism for restraining the initiation of egg formation until food and other aspects of the environment are all appropriate. Thus, in *Rhodnius*, where eggs are produced after each blood meal, hormonal regulation is necessary (Wigglesworth, 1936); whereas in the parthogenetic stick insect, *Dixippus*, in which feeding and egg production are continuous processes in the adult female, a hormonal control is not needed. Removal of the corpora allata does not influence yolk formation (Pflugfelder, 1937; other examples, see Engelmann, 1970). Since the synthesis of vitellogenin is an integral part of egg formation, there is no reason to doubt that its control too has not been under the same selection pressures.

The adult Cecropia silkmoth has all its nutrients provided by the feeding caterpillar and completes egg formation prior to adult emergence. The moth, in its short adult life, does little other than mate and lay its preformed eggs. A hormonal control mechanism for egg formation is therefore not necessary. Thus, the synthesis of vitellogenin is programmed as a component of metamorphosis rather than as, in most insects, an independent event. It should be pointed out that in several nonsaturniid species of Lepidoptera, in which egg formation depends upon feeding or drinking by the adults, the corpora allata are definitely required for vitellogenesis (see review by Wyatt, 1972; Doane, 1973), and the dependence of juvenile hormone for the induction of vitellogenin synthesis has been demonstrated in the Monarch butterfly (Pan and Wyatt, 1976).

The increase of vitellogenin observed by Blumenfeld and Schneiderman (1968) in intact pupae which received juvenile hormone extract requires some conjecture. Recall that the juvenile hormone extract is capable of stimulating the ecdysial glands to secrete ecdysone (Gilbert and Schneiderman, 1959; Williams, 1959). Ecdysone release can in turn cause the initiation of adult development (to become second pupae). Consequently, the synthesis of vitellogenin increases as it does during normal pharate adult development (Pan, 1971), and its concentration in blood rises.

I thank Dr. William H. Telfer for his advice and help; Dr. Robin A. Wallace for his generosity in letting me use his laboratory facilities and his farm to raise

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SUMMARY

1. The involvement of corpora allata and juvenile hormone in vitellogenin synthesis in the Cecropia silkworm was examined.

2. Allatectomy of diapausing pupae did not alter the pattern of ^{3}H-leucine incorporation into vitellogenin during their late pharate adult development.

3. Allatectomy of early fourth and fifth instar caterpillars did not prevent the appearance of vitellogenin in these operated animals when they had transformed into miniature pupae or pupal-adult intermediates.

4. Injection of a synthetic *dl*-JH into isolated diapausing abdomens did not stimulate the incorporation of ^{3}H-leucine into vitellogenin.

5. It is concluded that corpora allata and juvenile hormone are not required for the initial appearance and synthesis of vitellogenin in the Cecropia silkworm.

NOTE ADDED IN PRESS

Evidence that miniature pupae are indeed morphologically complete has now been obtained. Following one year storage at room temperature, one male miniature pupa that had been allatectomized in the early fourth larval instar developed and eclosed into a perfect miniature adult moth.

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CHEMORECEPTION IN THE BLUE CRAB, *CALLINECTES SAPIDUS*

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Chemoreception plays a dominant role in the feeding behavior of marine crustaceans. A logical first step in evaluating the precise role that sapid chemicals play in food gathering is to measure the animal's sensitivity to different levels of such substances. In this work behavioral criteria are utilized to measure the sensitivity of the blue crab, *Callinectes sapidus,* to a solution derived from natural food.

Studies using behavior to determine crustacean sensitivity to various amino acids and other substances have been few and have included only two studies of crustaceans other than the lobsters, *Homarus americanus,* (McLeese, 1970, 1974), *H. gammarus* (Mackie and Shelton, 1972; Mackie, 1973) and *Panulirus argus* (Levandowsky and Hodgson, 1965). In one other study, Fuzessery and Childress (1975) have compared the chemosensitivity of five crustaceans, the mysid, *Gnathophausia ingens,* the galatheid crab, *Pleuroncodes planipes,* the anomuran crab, *Pagurus hirsutiusculus,* the carid shrimp, *Spirontocaris taylori,* and the brachyuran crab, *Cancer antennarius.* Hindley (1975) has studied the ability of the prawn, *Penaeus merguiensis,* to detect amino acids. Where comparisons of sapid substances have been made, food extracts have generally been seen to be more effective in releasing feeding activity than even fairly complex synthetic mixtures (McLeese, 1970; Mackie, 1973).

The aims of this study were to observe the feeding behavior of the blue crab under laboratory conditions and select those components of the feeding repertoire that appeared to be most sensitive to the presence of sapid substances, utilize the selected behavioral components to determine the threshold concentration at which a food extract is detected, and examine the effects of food deprivation upon the detection threshold.

MATERIALS AND METHODS

After preliminary observations of the normal feeding behavior of the blue crab, *Callinectes sapidus,* a chemosensory testing apparatus was constructed in order to observe the response of crabs to various levels of sapid solutions. After numerous trials with the apparatus, two experiments were carried out: the first to establish thresholds for the detection of sapid material and the release of feeding, and the second to study the effect of food deprivation upon the thresholds.

For the preliminary observations and the first experiment, blue crabs, *Callinectes sapidus,* collected with dipnets and traps from the Shrewsbury River and northern Barnegat Bay, New Jersey, were transported in separate aerated containers in order to minimize handling stress. Mating pairs were not separated during transport or laboratory holding. The water at the site of the crabs' capture was 20.9° C (±2.4 s.d.) and 21.7‰ (±1.5 s.d.) For the second experiment, an unexpected early winter necessitated trawls of less than ten minutes in the Navesink River and

Sandy Hook Bay in order to obtain crabs in sufficient number. The crabs were carefully sorted into separate containers for transport. Capture by trawling is not as desirable as capture by less stressful methods, but short trawls and careful net handling considerably reduce handling stress. The water in the Navesink River was 9.2° C and 20.6‰.

At Sandy Hook Laboratory, a maximum of 72 crabs was maintained under natural photoperiod in a rectangular tank ($4.8 \times 1.8 \times 0.5$ m) with a sand bottom. Salt water from Sandy Hook Bay was recirculated through a gravel-sand-oyster shell filter before perculation through the sand on the tank's bottom with new water continuously added to maintain water quality. Both mating pairs and molting individuals were isolated. An *ad libitum* diet of blue mussels, *Mytilus edulis,* was provided. Crabs began to feed usually within hours of capture. During the first experiment from July to September, 1976, the temperature and salinity of water in the tank were 21.2° C (±0.7 s.d.) and 23.6‰ (±2.4 s.d.), respectively. During the second experiment, from November 1976 to January 1977, the crabs were gradually brought to and held at 20.6° C (±1.2 s.d.) and 23.9‰ (±0.4 s.d.)

The chemosensory testing apparatus was designed to present the crab with a sapid solution and to observe the response. Individual crabs were tested while isolated in 3.25-liter polystyrene chambers covered with white translucent plexiglass. Sea water passed through wound cellulose filters and a heat exchanger before entering a header tank with a gravel-sand-oyster shell filter. Water siphoning from the header tank entered each chamber *via* two lengths of plastic tubing, one carrying the main flow and the other used for adding the experimental solution. Both flows mixed as they entered the chamber. Flow rates were adjusteed to between 0.6 to 1.0 liter/min. A blind with view ports surrounded the water table upon which 18 chambers were arranged in a staggered line. Trials with dye solutions showed that 20 ml of solution injected within five sec into the inflowing water would disperse rapidly and completely through each chamber. To determine a dilution factor for estimating effective concentrations within a chamber, solutions of methylene blue were injected and the optical densities of water samples from the downstream end of the chamber compared with those of standard dilutions of the dye solution in a spectrophotometer at 663 nm. The maximum concentration occurred in the chamber between 0.75 and 1.0 minute injection of the solution. The maximum dye concentration within the chamber was 5.25×10^{-3} times the original concentration for a 1.07 liter/min flow rate and 4.85×10^{-3} times the original concentration for 0.60 liter/min flow rate. Using these measurements, estimated effective chambers concentration of the experimental solutions throughout this study was calculated by multiplying the concentration of the injected solution by 5×10^{-3}.

The sapid solution presented to blue crabs was a seawater solution of freeze-dried extract of hard clam, *Mercenaria mercenaria.* Preparation as well as chemical analysis of the freeze-dried clam extract (FDCE) was performed by the Southeast Utilization Research Center of the National Marine Fisheries Service. The dry weight composition of the FDCE was 53.7% protein, 5.9% fat, 7.9% ash, and 32.7% undetermined. Most of the amino acids comprised between one and two per cent of the FDCE dry weight. For the most abundant amino acids the dry weight composition was 4.2% taurine, 4.1% glutamic acid, and 3.2% aspartic acid.

On the day preceding each four-day period of testing, a stock FDCE solution was prepared as follows: first, a quantity of FDCE was ground in a mortar until powdered; secondly, a weighed portion of the powdered FDCE was mixed with sea water that had been filtered through a 0.4 μm membrane, and the resultant solution stirred for two hours with a magnetic stirrer; thirdly, the solution was filtered through tared Whatman No. 4 and GF/C filter paper; and fourthly, the concentration of the stock FDCE solution was corrected for the loss of the filtrant, which averaged 28.8% (±2.3) of the initial FDCE weight.

After preparation, the stock FDCE solution was refrigerated, and dilutions were made with membrane-filtered sea water approximately one hour before each day's testing.

Observation of an individual crab commenced at least one minute prior to injecting a FDCE dilution. The crab's posture and activities, presence of feces and regurgitated shell, and the extent of gill bailing were noted. Then, with the observer blind to the identity of the dilution, the FDCE was injected and observations of the crab's behavior recorded at 0.5-minute intervals for three minutes. Behavior was scored on the basis of criteria that were developed after observation of normal feeding and numerous trials in both experimental and holding tanks. A sharp increase in the antennule flicking rate accompanied by abrupt onset of continuous and vigorous gill bailing, all occurring within 1.5 minutes after FDCE introduction and continuing for at least 1.0 minute after onset, indicated detection. Feeding was considered to begin with chelae probing.

In the first experiment crabs were placed into the chambers between 1100 and and 1200 and tested the following day between 0900 and 1100. For 15 days, 18 blue crabs per day were presented with five dilutions of a 1.5 g/liter FDCE solution and a control of membrane-filtered sea water. The order of presentation and choice of dilution were taken from a random number table, except that the crabs that were active were passed over until they became still. Individual crabs were not retested without at least a two-day residence in the holding tank. Molting and mating crabs were not tested.

In the second experiment for four days, 18 crabs per day were tested as in the first experiment except that the number of dilutions was expanded from five to eight in order to present lower FDCE concentrations. Then, to investigate the effect of food deprivation on the detection threshold, the crabs were held without food for six days and retested in the same manner as before.

Regression analysis (Draper and Smith, 1966) was used to estimate the detection threshold. The threshold concentration was taken to be the concentration at which 50% of the crabs responded and was calculated from the regression equation relating the percentage of crabs responding and the logarithm of the FDCE concentration.

RESULTS

Normal feeding behavior and the selection of response criteria

Blue crabs would begin searching for food when juice from chopped clams, *Mercenaria mercenaria,* was dripped into an aquarium. A crab would rise from its resting or buried position and walk with its chelae extended and held just above the sand surface. Progress would be halting because the dactyls would move in arcs over the sand and occasionally probe beneath its surface. If a dactyl or chela contacted a piece of clam, the clam was quickly scooped inward and forward by the dactyls or grabbed by one chela and brought to the mouth to be torn by the maxillipeds and ingested. In its search a crab would walk over and away from a clam portion if neither a dactyl nor chela happened to contact the clam.

Crabs on an *ad libitum* diet of mussels would probe the mussel pile with dactyls and chelae and then separate one mussel from the rest by cutting the byssus threads with a chela. The crab would crack the shell with a chela and pry open the valves with both chelae as one would open a book. The chelae would then bring the opened mussel or bits of it to the mouth and hold it as the maxillipeds scraped the tissue from the shell. Large pieces of shell not ingested would be spit out after being scraped clean. The whole sequence typically would take three to five minutes with most of the time being spent in separating one mussel from the others.

During preliminary observations in the testing apparatus after the introduction of the FDCE, a sequence of behaviors was observed, the extent of which depended on the FDCE concentration. At high FDCE concentrations (10^{-2} to 10^{-4} g/liter), an increase in the rate of antennule flicking and the beginning of or increase to continuous gill bailing occurred and always preceded feeding and grooming behaviors. A rise in posture and the gaping and occasional labiating of the maxillipeds closely followed the antennule flicking and gill bailing. As antennule flicking and gill bailing continued, the crabs moved the dactyls in arcs touching the bottom. Such dactyl searching was preceded or accompanied by movements of the chelae probing toward the bottom. Crabs would attempt ingestion of shell bits or feces by bringing the material to the mouth with the chelae and then either spitting out the material or dropping it. Grooming usually followed feeding movements. The body was groomed by rubbing and picking motions of the dactyls and chelae, and the buccal area, by picking with the dactyls and chelae and rubbing with the palps. The palps also groomed the dactyls and chelae as well as the eyes, antennules, antennae and other mouthparts. Occasionally during grooming, an individual probed with the chelae or attempted ingestion, but usually grooming continued uninterrupted. Grooming entailed much rising and settling but little or no walking. At the highest FDCE level (10^{-2} g/liter) an abrupt rise in posture, immediate defecation, and vigorous grooming sometimes followed FDCE presentation. Attempted ingestion after defecation was rare and occurred usually near the end of observation.

At intermediate FDCE concentrations (10^{-4} to 10^{-8} g/liter) the full sequence was not observed. After walking and dactyl searching, the crab resumed a standing or resting position. Often instead of food search movements, grooming followed the initial increases in antennule flicking and gill bailing.

At low FDCE concentrations (10^{-8} to 10^{-12} g/liter) only the increase in the rate of antennule flicking and the onset of continuous gill bailing occurred. Sometimes there was a rise in posture, but no subsequent feeding behavior followed. Instead the crab settled into a resting posture, and the antennule flicking and gill bailing gradually returned to their initial state.

Because the increase in antennule flicking and gill bailing always preceded any feeding or grooming behavior and occurred alone at low FDCE levels, it appeared that the behavior of the antennules and gill bailers indicated detection of the FDCE and could be used to distinguish the levels of FDCE at which detection occurs from those levels at which food searching and gathering is released. To make this distinction the first experiment was performing using criteria for chemical detection and response with feeding movements, which were chosen from the behavioral sequences described above and which were defined in the Methods section.

The threshold for detection of FDCE solutions

The percentages of crabs detecting and responding with the feeding movement, chelae probing, were plotted against the logarithm of the estimated maximum FDCE concentration to which the crabs were exposed (Fig. 1). The FDCE concentrations at which 50% of the crabs detected the FDCE were calculated from the regression equations in Table I. Because the number of points where crabs exhibited feeding behavior was not adequate to construct a regression equation, the 50% threshold concentrations were estimated graphically (Fig. 1).

In the first experiment the feeding threshold was approximately 0.5 g/liter. In the second experiment crabs on *ad libitum* diet showed a feeding threshold of 10^{-2} g/liter and crabs deprived of food for six days had a lower feeding threshold of 10^{-3} g/liter.

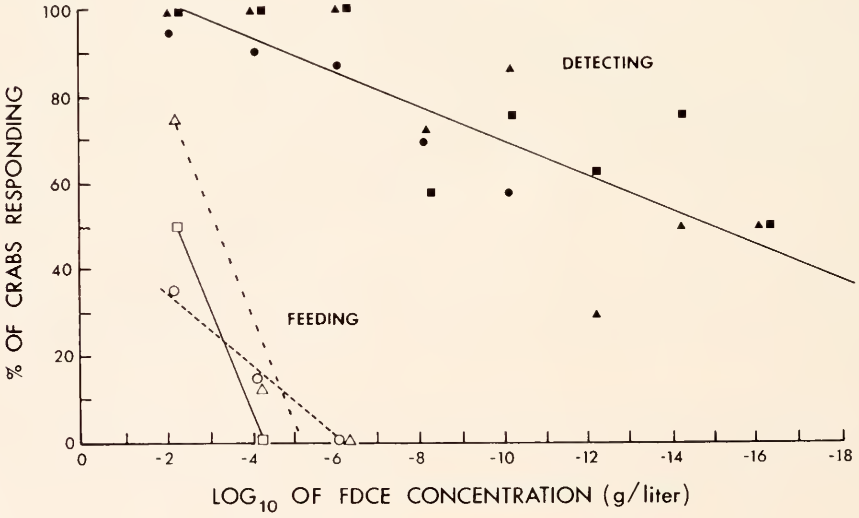

FIGURE 1. The percentage of blue crabs either detecting the FDCE or exhibiting feeding behaviors as a function of the logarithm of FDCE concentration: solid shapes indicate detecting; open shapes, feeding; circles represent the first experiment; squares, the second experiment with crabs on an *ad libitum* diet; triangles, the second experiment with crabs deprived of food for six days. In the first experiment each point represents 45 trials; in the second, 8 trials. For the first experiment the percentage of crabs detecting the control sea water was 34% (47 trials); for the second, 25% (8 trials) for crabs on an *ad libitum* diet and 38% (8 trials) for crabs after six days' food deprivation.

TABLE I

Regression equations relating the percentage of crabs detecting the FDCE to the logarithm of the FDCE concentration: Y = percentage of crabs detecting; $X = \log_{10}$ [FDCE].

	Equation	R^2	95% Confidence intercept	Limits slope	[FDCE] at which 50% detection is predicted g/liter
First experiment	$Y = 109.78 + 4.94X$	0.960	±12.77	±1.89	7.9×10^{-13}
Second experiment					
With food	$Y = 108.70 + 3.39X$	0.650	±25.42	±2.47	4.8×10^{-18}
Without food	$Y = 116.99 + 4.76X$	0.707	±31.40	±3.06	8.5×10^{-15}
Combining with and without food	$Y = 109.40 + 3.74X$	0.737	±22.96	±2.23	1.4×10^{-16}
Combining all experiments	$Y = 109.50 + 3.92X$	0.664	±12.80	±1.34	6.5×10^{-16}

The detection thresholds in both experiments were several orders of magnitude lower than those for feeding. The regression equations for detection by crabs deprived of food and by crabs on an *ad libitum* diet did not differ significantly in slope or intercept and, therefore, were combined (Table I). The regression equation for detection in the second experiment also did not differ in slope or intercept from that of the first, but did show more variability. Because detection equations did not differ from experiment to experiment or with or without food, they were combined into one equation.

From this resultant equation the FDCE concentration at which 50% (±5.14 est. s.e.) of the crabs detect the FDCE proved to be 6.5×10^{-16} g/liter. Back calculation of the 95% confidence limits about the regression line indicated that the detection threshold fell between 10^{-13} and 10^{-18} g/liter. The detection threshold was from 10^{10} to 10^{17} times lower than the feeding threshold and did not change after food deprivation for six days.

DISCUSSION

With ablation experiments Hazlett (1971) has demonstrated that the antennules of *Callinectes sapidus* and most other decapods examined function as distance chemoreceptors. We have found that close attention to the antennular motion allows one to discern when a sapid substance is detected. The gill bailing accompanying the elevated antennule flicking rate increases the rate at which water flows out of the buccal area and around the antennules. The increased antennule flicking and gill bailing presumably magnifies the rate at which water is sampled and thus can be viewed as an active search by the crab for more information about its chemical environment.

In these experiments the linear regression line used to estimate thresholds is an approximation of the relationship between the percentage of crabs detecting the FDCE and the logarithm of its concentration. One can reasonably expect the extreme upper portion of the curve to become asymptotic to 100% because at very high FDCE concentration almost all the crabs will detect the FDCE. Similarly, because reactions of crabs to the control sea water indicate that a certain low percentage of the crabs will react to any change in the incoming water flow, it is reasonable to expect the extreme lower portion of the curve to become asymptotic to some control value. Because the departure from linearity occurs at the extremes of the curve, the regression line can still be used to estimate the 50% threshold.

Fuzessery and Childress (1975) have discussed the postulate that among decapods contact chemoreception occurs at the less chemosensitive dactyls, while distance chemoreception occurs at the more sensitive antennules. The postulate suggests that the difference in feeding and detecting thresholds seen in *C. sapidus* may derive from a difference in chemosensitivity between dactyls and antennules. If the release of feeding behavior in *C. sapidus* requires stimulation of contact receptors on the dactyls or buccal region, this requirement may account for the high feeding threshold in *C. sapidus.*

The fact that low concentrations of sapid material release antennule flicking and gill bailing does not indicate, however, whether these behaviors constitute a reflex or involve integration at higher centers. The animal's history, both the evolutionary history of its species and the particular history of the individual under natural conditions, influences at what level of sapid solution the animal will show more complex behaviors such as food gathering. The question remains, therefore, whether the low levels of sapid material (10^{-15} g/liter FDCE) provide sufficient information for the occurrence of complex behavior or whether such low levels only serve to prime or alert the animal.

Apparently, only Fuzessery and Childress (1975) have noted grooming as an alternative response to sapid solutions, as seen in *C. sapidus.* Because the blue crab rubs the palp over the antennule, chelae, dactyls, and mouthparts, which have high densities of chemoreceptors, one suspects that such grooming serves to clean the chemoreceptors. For the hermit crab, *Pagurus alaskensis,* Snow (1973) has found that antennular wiping by the endopodites removed debris from the aesthetasc hairs.

The startle response with subsequent defecation and vigorous grooming observed with high FDCE levels may be an avoidance reaction. In the spiny lobster, *Panulirus argus,* Levandowsky and Hodgson (1965) have observed avoidance responses to high levels of amino acids and amines that elicited feeding at lower levels. In this study, if such startle responses to high FDCE levels were equated to feeding responses, the estimated feeding threshold would drop in the first experiment to 10^{-2} g/liter, but would not change in the second experiment.

Comparison of thresholds for *Callinectes sapidus* with those of other crustaceans is difficult because the response criteria, sapid substances, and food deprivation schedules vary among investigations. Nevertheless, somewhat comparable results exist for five other crustaceans. For the lobster, *Homarus americanus,* McLeese (1974) has estimated the threshold concentration at which an extract of cod muscle released upstream walking to be 3×10^{-5} g/liter. Using the regression equation of Mackie (1973) for the lobster, *H. gammarus,* the threshold at which a lipid-free squid mantle extract released gathering motions of the chelate pereiopods and upstream walking was calculated to be 2.1×10^{-6} g/liter. Our regression of the data of Fuzessery and Childress (1975) has shown the threshold concentrations for the release of feeding motions by an equimolar mixture of three amino acids to be 6.8×10^{-3} g/liter for *Pagurus hirsutiusculus,* 2.3×10^{-7} g/liter for *Pleuroncodes planipes,* and 3.0×10^{-9} g/liter for *Cancer antennarius.* Because mixtures of amino acids alone have been found to be less attractive than food extracts or complex synthetic mixtures (Shelton and Mackie, 1971; Mackie, 1973), the last three crustaceans would perhaps have shown lower thresholds if they had been assayed with a food extract instead of an amino acid mixture. The major difference between this study of *C. sapidus* and those of the other crustaceans is that the response criteria for the other studies were limited to feeding motions, while for the blue crab an additional set of criteria was applied. When comparing the thresholds established with feeding motions, *C. sapidus* appears less sensitive to sapid materials than the other crustaceans except for *P. hirsutiusculus,* but the fact that the detection threshold of 10^{-15} g/liter for *C. sapidus* is lower than both its feeding threshold and those of the other crustaceans evinces that the behaviors indicating detection, *i.e.*, increased antennule flicking and gill bailing, are more sensitive response criteria than the feeding motions generally used. The blue crab's low detection threshold also suggests that crustaceans may detect lower levels of sapid substances than previously believed.

Mackie and Shelton (1972) found that, after nine days' starvation, the feeding threshold of *Homarus gammarus* decreased from 10^{-4} to 10^{-6} g/liter. After six days of food deprivation, the feeding threshold of *C. sapidus* dropped from 10^{-2} to 10^{-3} g/liter FDCE, but the detection thresholds did not change. Thus, food deprivation lowers the feeding threshold, but, at least for *C. sapidus*, does not bring the animal to respond at its limit of chemical detection or depress that limit. The remaining questions are whether food deprivation lowers the feeding threshold to some consistent degree and whether food deprivation longer than six days influences the detection threshold.

The increased antennule flicking rate and gill bailing may indicate not just the detection of sapid chemicals but the sensing of any chemical discontinuity in the crab's environment. If so, the behavioral assay based on the observation of antennular behavior could be used to investigate the detection of other chemicals important in the crab's life history, *e.g.*, those playing a part in habitat selection or social behavior. Also the reasonable assumption that the chemical milieu varies geographically leads us to expect that detection thresholds based upon sensing chemical discontinuities also vary geographically.

We wish to thank Dr. Garfield Biddle and his colleagues at the Southeast Utilization Research Center of the National Marine Fisheries Service for providing the facilities to prepare the freeze-dried clam extract and performing chemical analysis of the extract. We also wish to thank our laboratory colleagues, A. D. Martin, C. Samet, A. Bejda, and A. Studholme, for their aid through various phases of the research and E. Lyszczek and D. Atkin for aid in collecting animals.

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SUMMARY

1. An increase in the rate of antennule flicking and gill bailing upon presentation of sea water solutions of a freeze-dried clam extract indicated detection of sapid substances by the blue crab, *Callinectes sapidus.*

2. The threshold concentration at which crabs detected the sapid solution was 10^{-15} g/liter. Feeding behaviors were released at higher concentrations, 10^{-1} to 10^{-2} g/liter.

3. Food deprivation for six days lowered the threshold for feeding behaviors but did not affect the detection threshold.

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AGGRESSIVE FUNCTION AND INDUCED DEVELOPMENT OF CATCH TENTACLES IN THE SEA ANEMONE *METRIDIUM SENILE* (COELENTERATA, ACTINIARIA)

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Catch tentacles, found near the mouth in some individuals of the sea anemone *Metridium senile*, are more opaque than the regular tentacles and conspicuously larger. In anemones about 6 cm in expanded column height, representative dimensions of the regular tentacles are 0.5 × 7.0 mm, while noninflated catch tentacles measure about 3.0 × 20.0 mm. Catch tentacles are capable of expanding to several times their resting length and breadth, reaching dimensions of 5.0 × 120.0 mm. When thus expanded, a catch tentacle repeatedly extends and retracts, touching its tip to the substratum. These movements were first mentioned by Gosse (1860) in several British anemones. Carlgren (1929) named the large, inflatable tentacles *Fangtentakeln* and showed them to have a different cnidom from the other tentacles of the anemone in the species *Diadumene cincta, D. neozelanica,* and *D. kamerunensis.* Catch tentacles are now known to occur in at least some species in six families of acontiate anemones: Diadumenidae, Sagartiidae, Metridiidae, Isophelliidae, Sagartiomorphidae, and Haliplanellidae (Williams, 1975).

The catch tentacles in specimens of *Metridium senile* from the central California coast were described by Hand (1955). He reported that specimens with catch tentacles formed less than one per cent of the intertidal populations, did not occur as isolated individuals, and showed no obvious pattern in their distribution. He found the catch tentacles to have a strikingly different cnidom than the regular tentacles. The main thrust of Hand's work was taxonomic, and the few observations he made on the activities of catch tentacles suggested they might aid in feeding.

Williams (1975), in studies on *Haliplanella luciae* and *Diadumene cincta,* found that food and other materials did not adhere to the catch tentacles and that the tentacles were not brought to the mouth. Williams cited observations of P. R. G. Tranter of the Plymouth Marine Laboratory that in the anemones *Cereus pedunculatus, Sagartia elegans* and *S. troglodytes,* the catch tentacles were used offensively against members of their own species from different localities and against other species. When the tip of an expanded catch tentacle contacted another anemone, it adhered to it; later the catch tentacle formed a constriction and broke here, leaving its tip attached to the victim. The catch tentacles of these species did not adhere to food. Williams (1975, p. 244) further noted that "*Metridium senile* and *Diadumene cincta,* although possessing catch tentacles, were not observed exhibiting aggressive behavior by Tranter."

Metridium senile reproduces asexually by pedal laceration (Stephenson, 1935), thus forming clones of a few to many hundreds of individuals. Clonemates are remarkably alike and are easily distinguishable from members of other clones by body color, most commonly white or various shades of brown, and by the presence or absence of a pattern of white banding on the bases of the tentacles. Clones are formed in the anemone *Anthopleura elegantissima* by fission and individuals exhibit aggressive behavior toward members of other clones (Francis, 1973a, b).

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The purpose of the present study is to re-examine catch tentacle function in *Metridium senile*.

MATERIALS AND METHODS

This study was conducted at the Hopkins Marine Station of Stanford University, from September, 1975, through January, 1976. Extensive populations of *M. senile* grow nearby on the pilings of Wharf No. 2, Monterey, California. Field observations and collections were made using SCUBA gear. Specmens returned to the laboratory were kept in plastic pans provided with running sea water at 12–13° C, each pan containing only members of one clone. Experiments were conducted after the anemones had attached by the pedal disc and had expanded the tentacle crown, usually 24–48 hours after collection. Anemones were used in behavioral observations for one week. The anemones observed ranged in size from approximately 5 to 10 cm in expanded column height.

Details of experimental protocols accompany the description of each experiment in Results.

RESULTS

Responses to food

To determine if *Metridium senile* uses its catch tentacles in feeding, living crustaceans (the copepod *Tigriopus californicus* and larvae of the brine shrimp *Artemia salina*), were offered as food. Individual prey animals were held by the abdominal segments with fine forceps. Prey animals were touched first to a catch tentacle, secondly to a regular tentacle, and thirdly to a catch tentacle. The same procedure was followed in fifteen animals in which one or more catch tentacles were inflated, touching the prey animal to both inflated and resting catch tentacles, and in fifteen in which the catch tentacles were not inflated. Each prey animal captured was replaced by another.

In no case did the catch tentacles adhere to the food offered, whereas it was always captured by the regular tentacles. When touched with a prey animal, the catch tentacles retracted slightly, just as they responded to similar touches with a clean probe. Not only did the prey animals not adhere to the catch tentacle, but they were able to swim away after repeated contacts with the sides and tip of a catch tentacle. In contrast, prey animals presented to the regular tentacles immediately became attached to the tentacle and were immobilized. The tentacle contracted, and with others surrounding it, curled and bent toward the mouth where the prey was ingested. Following active feeding involving regular tentacles, the catch tentacles still did not capture prey animals. Only once did a detrital particle adhere to an expanded catch tentacle. It was not delivered to the mouth and later came free.

These observations show that the catch tentacles of *Metridium senile* are not used in feeding. They did not capture prey and did not exhibit any of the characteristic feeding movements of the other tentacles.

Distribution of individuals with catch tentacles within local populations

Considering the possibility that catch tentacles might be used offensively against nonclonemates, the distribution of specimens of *Metridium senile* with catch tentacles was reinvestigated. The dense subtidal populations at depths of approximately 2 to 25 feet on wharf pilings at Monterey provided the most favorable units for study. Preliminary observations indicated that specimens of *M. senile* with catch tentacles were usually found along the margins of clones opposing different clones. This was documented by a series of color photographs of twenty-five clones showing the following three types of sites: first, the borders of clones where individuals of one clone were situated within approximately 10 cm (the length of an expanded catch tentacle) of anemones of another clone; secondly, the centers of clones, where anemones were surrounded by clonemates and were not within reach of individuals of other clones; and thirdly, the edges of clones where the outermost anemones those without catch tentacles were counted in all photographs for each of the three were not within reach of individuals of a different clone. Anemones with and types of sites (Table I).

Specimens of *Metridium senile* with catch tentacles are conspicuously abundant at borders separating adjacent clones, and very scarce elsewhere. While 22.6% (355/1523) of the 1523 anemones examined possessed catch tentacles, this is not an unbiased estimate of the abundance of anemones with catch tentacles in the entire population, because greater attention was focused on the borders where members of two clones were in near contact. Of those anemones with catch tentacles, 96% (339/355) were at these borders, and 77% (339/441) of the border anemones had catch tentacles. Border anemones lacking catch tentacles were usually the smallest individuals. G-tests (Sokal and Rohlf, 1969) showed no significant differences between numbers of individuals with catch tentacles at the centers of clones and those at the edges of clones where there was no contact with nonclonemates. In contrast, differences between numbers of individuals with catch tentacles in border and nonborder (center plus edge) areas were highly significant ($P < 0.005$).

TABLE I

Distribution of specimens of M. senile *possessing catch tentacles, with respect to their positions within clones.*

Positions of anemones	Number of anemones	
	With catch tentacles	Total observed
At clone margin bordered by another clone	339	441
At center of clone	11	857
At clone edge not adjacent to another clone	5	225
	355	1523

The separation of clones can be quite marked, with an anemone-free zone 7–12 cm wide extending between the clones, and with specimens bearing catch tentacles lining the borders (Fig. 1). In other cases different clones occur in close proximity with no distinct anemone-free border zone, but neighboring members of both clones possess catch tentacles. This is the case where a small number of anemones of one clone are found surrounded by members of a larger clone. Among the 1523 anemones enumerated, only eleven anemones with catch tentacles were seen in the centers of clones where no adjacent nonclonemate could be identified.

Many specimens of *M. senile* have catch tentacles where clones of *M. senile* and *Anthopleura elegantissima* meet at intertidal levels on the pilings. The two species are not separated by a wide anemone-free zone.

In summary, field observations indicate that specimens of *Metridium senile* with catch tentacles occur in circumstances where nonclonemates are in close proximity. This suggests the occurrence of catch tentacles may be determined by the presence of nonclonemates.

Behavioral studies on the role of catch tentacles

The facts that catch tentacles in *Metridium senile* are not used in feeding and that they occur predominantly in individuals along borders separating two different clones suggest that they function in aggression against genetically different anemones. Field and laboratory observations were made to ascertain whether aggression is exhibited.

Catch tentacles were first observed in undisturbed natural populations to get indications of their use. In the field, an occasional anemone was seen with one or more catch tentacles inflated and moving with the current. When the sides and tip of the inflated tentacle of one anemone brushed any body surface of its clonemates, there was no response by either anemone. When the catch tentacle tip contacted a nonclonemate, approximately 1 cm of the tip attached to the nonclonemate, which contracted locally. The catch tentacle remained attached and after two to five minutes began to withdraw and deflate, breaking about 1 cm behind the tip. This sequence was sometimes repeated with another catch tentacle on the same anemone. Of the numerous catch tentacles observed expanded in the field during the course of the study, only five instances of contact with nonclonemates were witnessed.

More detailed studies of catch tentacle behavior were carried out in the laboratory. Anemones with and without catch tentacles, collected from several clones, were kept in holding pans in the laboratory. Studies were made of the behavioral interactions between over forty pairs of clonemates and fifty pairs of nonclonemates, including some in which *Metridium senile* was paired with *Anthopleura elegantissima*. Interactions between pairs were recorded in the following laboratory situations: undisturbed anemones in pans containing members of only one clone, and in pans containing anemones of more than one clone; and anemones settled on movable glass squares. In the latter case, expanded anemones were gently moved so that the tips of the feeding tentacles of the two animals were either just out of contact (and allowed to contact naturally), or they were carefully moved so that the anemones experienced tentacle-tip contact. No differences in behavior were seen which appeared attributable to the method by which initial tentacle con-

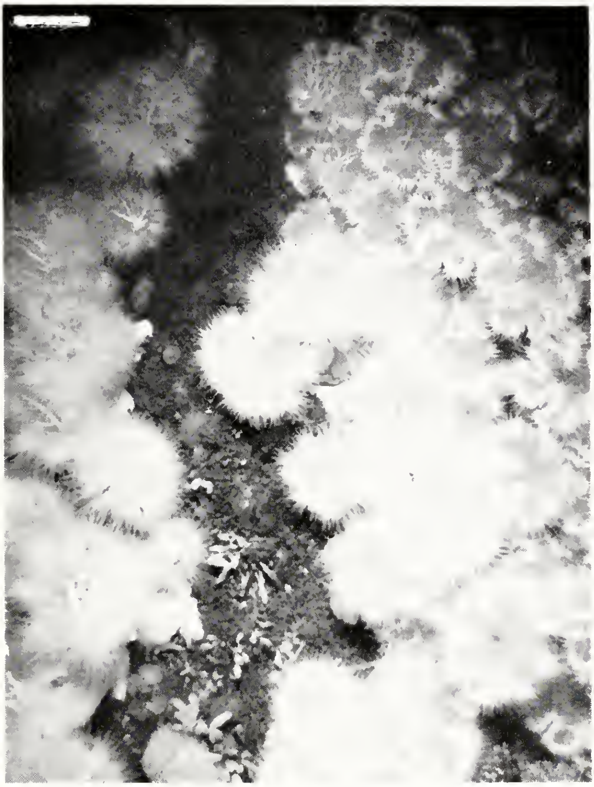

FIGURE 1. Two adjacent clones of *M. senile* on a wharf piling at Monterey, California. The clones are separated by an open corridor bordered by anemones with catch tentacles. Scale bar equals 3 cm.

tact was established by the pair. Anemones on glass squares were out of contact with all other anemones for a period of at least an hour.

Results of the experiments are summarized in Table II and Figure 2, while Figure 3 illustrates a typical sequence of behavior between two nonclonemates with catch tentacles following mutual contact of feeding tentacle tips. In all cases of clonemate and nonclonemate contact, both anemones responded to this contact with slight withdrawals of the tentacles touched, and a slight swelling of these and other feeding tentacles particularly in the area of contact. Tentacle retractions of both anemones were more pronounced upon contact between nonclonemates and were especially violent when specimens of *M. senile* contacted other species (*e.g., Anthopleura elegantissima*).

After numerous feeding tentacles had made mutual contact (Fig. 3A), each individual of the pair of *M. senile* began bending its column, first away from, and then toward, the area of contact with the other anemone (Fig. 3B). This bending brought the feeding tentacles of the two animals repeatedly in and out of contact. The period of bending varied from ten minutes to four hours, but typically lasted about twenty minutes.

TABLE II

The behavioral interactions following contact between the tips of feeding tentacles in anemone pairs, including individuals with and without catch tentacles (CT) in paired clonemates, paired nonclonemates, and M. senile (M.s.) *paired with* Anthopleura elegantissima (A.e.). *Behavior of each pair is followed through each distinguishable unit of the interaction. Anemones had been out of contact with all others for at least an hour. Ratios represent the number of pairs in which a positive response was recorded out of the number of pairs tested. An asterisk marks each case where an inflated catch tentacle was made to contact another anemone without previous feeding tentacle tip contact.*

Features of the behavioral interaction	Responses of anemones of interacting pair							
	M.s., clonemates			*M.s.* nonclonemates			*M.s. vs. A.e.*	
	Both without CT	a) With CT b) Without CT	Both with CT	Both without CT	a) With CT b) Without CT	Both with CT	*M.s.* without CT	*M.s.* with CT
Tentacles retract on tentacle tip contact between pairs members	5/5	a) 6/6 b) 6/6	20/20	5/5	a) 5/5 b) 5/5	25/25	*M.s.* 3/3 *A.e.* 3/3	*M.s.* 6/6 *A.e.* 6/6
Column bends following the above	5/5	a) 6/6 b) 6/6	20/20	5/5	a) 5/5 b) 5/5	25/25	*M.s.* 3/3 *A.e.* inflates acroraghi	*M.s.* 6/6
Regular tentacles applied to tentacle crown of other anemone	0/5 —	a) 0/6 b) 0/6	0/20	5/5	a) 0/5 b) 0/5	1/25	*M.s.* 3/3 *A.e.* 0/3	*M.s.* 0/6 *A.e.* 0/6
Catch tentacles expand	—	a) 6/6 b) —	16/20	—	a) 5/5 b) —	25/25	—	*M.s.* 5/6 *A.e.* —
Catch tentacle adheres to other anemone upon contact	—	a) 0/6 b) —	0/16 *0/10	—	a) 5/5 b) —	25/25 *10/10	—	*M.s.* 5/5 *A.e.* —
Victim's catch tentacles subsequently expand	—	—	0/16 *0/10	—	—	24/25 *10/10	—	—
Injury incurred	None	a) None b) None	None	Slight necrosis	a) None b) Necrosis	Necrosis	Necrosis	*M.s.* none *A.e.* necrosis

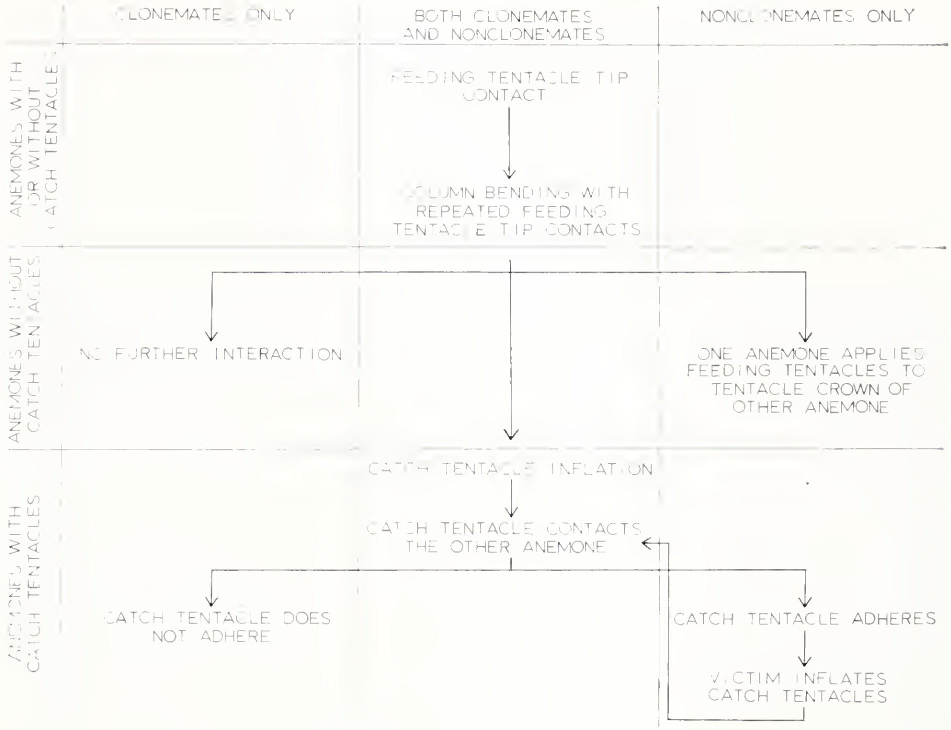
CLONEMATES ONLY
BOTH CLONEMATES AND NONCLONEMATES
NONCLONEMATES ONLY
ANEMONES WITH OR WITHOUT CATCH TENTACLES
ANEMONES WITHOUT CATCH TENTACLES
ANEMONES WITH CATCH TENTACLES
FEEDING TENTACLE TIP CONTACT
COLUMN BENDING WITH REPEATED FEEDING TENTACLE TIP CONTACTS
NO FURTHER INTERACTION
ONE ANEMONE APPLIES FEEDING TENTACLES TO TENTACLE CROWN OF OTHER ANEMONE
CATCH TENTACLE INFLATION
CATCH TENTACLE CONTACTS THE OTHER ANEMONE
CATCH TENTACLE DOES NOT ADHERE
CATCH TENTACLE ADHERES
VICTIM INFLATES CATCH TENTACLES

FIGURE 2. Pattern of behavioral units in anemone pair interaction following feeding tentacle tip contact between pair members which had not been in contact with other anemones for an hour or more. Pairs include individuals of *M. senile* with and without catch tentacles *versus* clonemates and nonclonemates with or without catch tentacles.

Of anemones without catch tentacles, clonemates ceased notable interaction at this point; they stopped bending and either remained in contact, or moved away. The bending behavior was observed in clonemates which had been separated from contact with other members of their clone for a period of hours. In nonclonemates, however, the column-bending became more exaggerated. Eventually, one of the pair brought a large portion of its tentacle crown down on the upper surface of the tentacle crown of the second anemone. This action was sometimes repeated. The second anemone ceased column bending and later showed slight necrosis on its tentacles. Following the encounter, one or both anemones moved out of contact.

Where one or both members of a pair, either clonemates or nonclonemates, of *M. senile* had catch tentacles, catch tentacle inflation always followed the period of bending with repeated feeding tentacle tip contacts (Fig. 3C). Usually only one, and sometimes two, catch tentacles inflated per individual. Most frequently, the catch tentacles of one indvidual inflated and contacted the second anemone before the second inflated its catch tentacles. Nearly simultaneous catch tentacle inflation in both anemones did sometimes occur. Inflated catch tentacles displayed considerable activity, extending and partially retracting, while repeatedly touching the tip to the surroundings.

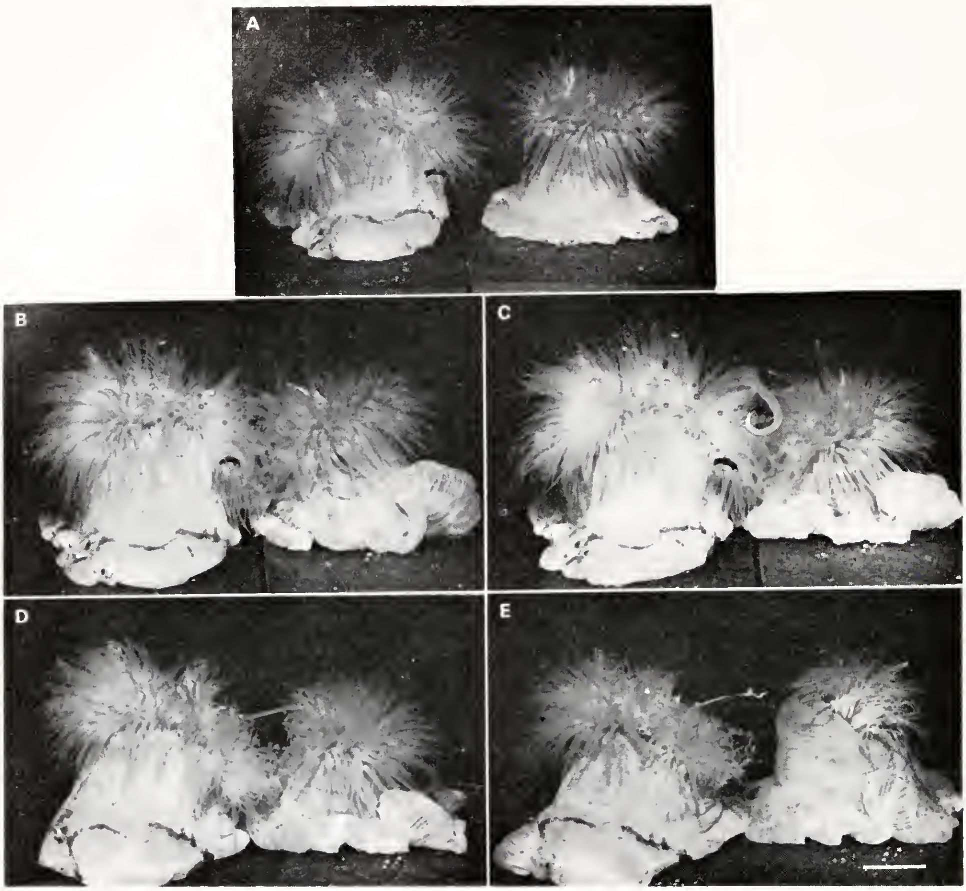

FIGURE 3. Sequence of behavior in two nonclonemates of *M. senile*, both with catch tentacles: a) feeding tentacle tips have come into contact; the white tips of two noninflated catch tentacles are visible in the anemone on the right, which begins to bend away from the point of contact; b) the anemone on the right now bends the column toward the site of initial contact; repeated column-bending motions of both anemones lead to repeated contacts of the feeding tentacles; c) an inflated catch tentacle from the anemone on the left has attached at several points, near and at its tip, to tentacles of the individual on the right, in which several catch tentacles are inflating; d) the attached catch tentacle of the left anemone has begun to deflate and withdraw; the victim on the right shows partial contraction of the tentacles in the area of catch tentacle adhesion; e) the catch tentacle of the left anemone has broken contact. The victim on the right has strongly contracted and is inflating several white-tipped catch tentacles. Scale bar equals 1.5 cm.

When the catch tentacle tip contacted a clonemate, no reaction by either anemone was observed. The catch tentacle often remained in contact with the clonemate for several minutes: it moved the tip over the clonemate's column, base, and tentacles, but it never adhered and eventually withdrew and deflated. The clonemates observed had been out of tentacle contact with other clonemates for a period of at least an hour.

When an expanded catch tentacle contacted a nonclonemate on either the tentacle crown or column, about 1 cm of the catch tentacle tip immediately adhered. The victim contracted sharply near the site of attachment, but did not withdraw completely. The aggressor sometimes moved the inflated unattached portion of the catch tentacle, enabling sites further from the tentacle tip to attach to the victim (Fig. 3C). After two to five minutes, the catch tentacle began to pull back and deflate (Fig. 3D), leaving the attached portions of the catch tentacle adhering to the victim. An obvious constriction sometimes formed 1 cm behind the tip, where breakage subsequently occurred (Fig. 3E).

In encounters between two nonclonemates both possessing catch tentacles, an attack by a catch tentacle of one animal led to the immediate expansion of the victim's catch tentacles (Fig. 3C, E). This inflation of one, or frequently several, catch tentacles took place in one to five minutes, sometimes while the aggressor's catch tentacle was still attached. In experimental situations where an inflated catch tentacle was made to contact a nonclonemate without previous mutual feeding tentacle contact between the two animals, the victim's catch tentacles also inflated. Up to nine successive attacks by one specimen of *M. senile* upon another were observed, each attack involving a different catch tentacle. More usually one to three attacks were made by each individual of a given pair of nonclonemates. One or both anemones eventually moved away. In all cases severe necrosis ensued where the catch tentacle tips remained attached to a victim, and in three anemones death followed several days later.

Anemones well separated from any contact with others sometimes inflated a catch tentacle. The stimulus for this is not known. On the other hand, the pedal discs of clonemates and nonclonemates were sometimes observed to be in contact for many hours without the catch tentacles ever inflating. In no case was a contact between pedal discs, or between the tentacles of one animal and the base of another, associated with catch tentacle inflation.

Contact of a catch tentacle tip with any portion of a genetically different anemone resulted in adherence of the tip and in contraction of the victim at the site of adherence, indicating nematocyst discharge from the catch tentacle tip. Catch tentacle contact with clonemates showed no indication of any nematocyst discharge.

Inducing the formation of catch tentacles

The occurrence of catch tentacles in individuals of *Metridium senile*, residing adjacent to nonclonemates of *M. senile* or to other anemones such as *Anthopleura elegantissima*, suggests that formation of catch tentacles is induced by contact between different anemones. In two clones separated by over 30 cm, no anemones had catch tentacles, except where a wanderer from one clone had come to rest next to the other clone. Four anemones immediately adjacent to the wanderer had several catch tentacles each. What appeared to be partially developed catch tentacles were seen in the field; the outermost anemones in a clone had numerous large and very opaque catch tentacles and the next few more central anemones had fewer, smaller, and more transparent catch tentacles.

When an anemone bears catch tentacles, these always occur in the one or two circles of tentacles nearest the mouth. The nematocysts of catch tentacles are strikingly different from those in the feeding tentacles. Holotrichs, atrichs, and microbasic amastigophores are found in catch tentacles. Spirocysts, and microbasic a and *b*-mastigophores constitute the bulk of the nematocysts in the feeding tentacles, with some basitrichs found near the bases of the tentacles (Hand, 1955).

In order to test the hypothesis that contact with nonclonemates induces formation of catch tentacles, seven individuals without catch tentacles were selected from each of two clones and were kept in running sea water in a small aquarium (14 × 14 × 10 cm). Under these crowded conditions, frequent tentacle contacts between nonclonemates were inevitable. Counts of the major types of nematocysts were made at the beginning of the experiment and after four, six, and nine weeks, in tentacles distant from the mouth, tentacles in the first circle surrounding the mouth which appeared unchanged, and tentacles of the first circle in which opacity increased. Tentacles were removed by grasping them near the base with fine forceps and pulling lightly, causing the tentacle to come free where joined to the oral disc. Tentacles were mounted in sea water on slides under a coverslip and gentle pressure applied to yield an even squash preparation. Preparations were examined at 1000× magnification. Differential counts of nematocyst types were made in five arbitrarily selected fields representing both the tip and base of the tentacles (Fig. 4, Table III).

A slight opacity of one first-circle tentacle was noticed in one individual after one week, and in four of the seven members of the same clone at two weeks. Visible signs of catch tentacle formation in some members of the second clone occurred after four weeks. At nine weeks, eleven of the fourteen individuals possessed catch tentacles. The number of catch tentacles per individual ranged from two to thirteen.

Figure 4 shows the loss of feeding tentacle nematocysts and the acquisition of catch tentacle nematocysts in developing catch tentacles over the nine-week confinement of two clones. Counts were made of the nematocysts: at four weeks, in one tentacle in each of three individuals of one clone; at six weeks, in one tentacle in each of seven individuals from both clones; and at nine weeks in one or two tentacles in all eleven anemones which possessed catch tentacles. In Table III, counts made at zero and nine weeks of the cnidoms of feeding tentacles, both close to and far from the mouth, and of the cnidoms of fully developed catch tentacles, are compared to cnidom counts in the tentacles nearest the mouth which increased in capacity in the confined clones at week nine. Counts were made of nematocysts in two feeding tentacles taken close to the mouth, and two taken far from the mouth, in each of four individuals in each clone at zero and nine weeks. The nematocyst types were counted in two fully-developed catch tentacles taken from each of four members of one experimental clone which already bore catch tentacles at week zero. The proportions of nematocyst types given in Figure 4 and Table III are all averages of counts taken at the tips and bases of the tentacles.

In the experimental animals, the mean percentages of the nematocyst types in the feeding tentacles (both peripheral tentacles and those nearest the mouth) did not vary substantially during the nine weeks; spirocysts constituted approximately 80% of the cnidom. Some tentacles in the first circle around the mouth showed an increasing opacity first near the tip, and later over the entire tentacle; this was accompanied by an increase in the length and width of the tentacle. Tentacles undergoing this change invariably contained catch tentacle nematocysts (atrichs and holotrichs) in varying stages of development, in addition to nematocysts char-

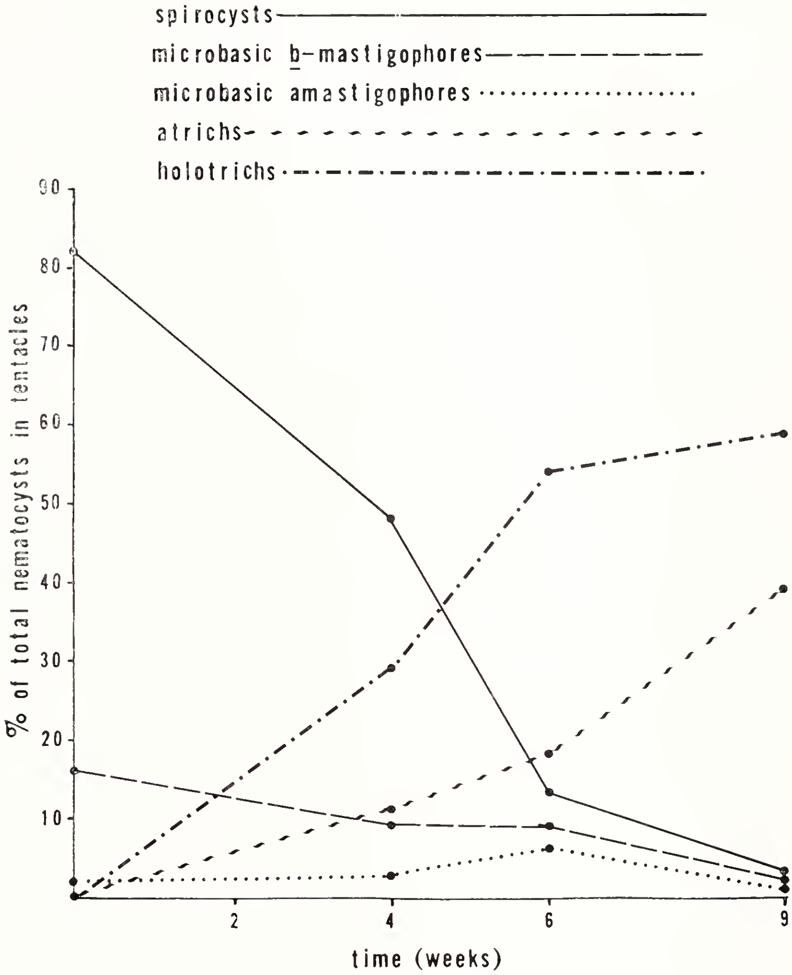

FIGURE 4. Average proportions of the several nematocyst types in tentacles of *M. senile*. Seven anemones without catch tentacles from each of two clones were confined for nine weeks such that frequent feeding tentacle contact was inevitable. Nematocyst counts were made of tentacles nearest the mouth at the time of first interclonal contact; the counts were repeated after four, six, and nine weeks in those tentacles nearest the mouth which showed visible increases in opacity. Data points are indicated with solid circles.

acteristic of feeding tentacles. The proportion of catch tentacle nematocysts increased throughout the nine weeks, as spirocysts and *b*-mastigophores declined in numbers and disappeared (Fig. 4). The proportions of the major nematocyst types in feeding tentacles (spirocysts and *b*-mastigophores) and in catch tentacles (atrichs and holotrichs) in the experimental animals were compared between week 0 and week 9 in a Kendall Rank Correlation test (Sokal and Rohlf, 1969). The test gave a highly significant negative correlation, with $P < 0.001$. The proportions of different types of nematocysts in the developing catch tentacles after

Table III

Average proportions of the several nematocyst types in tentacles of M. senile. *Nematocyst counts were made of fully developed catch tentacles in individuals of one clone at week 0. Seven members of this clone and seven from a second clone, all lacking catch tentacles, were confined for nine weeks where frequent feeding tentacle contacts were inevitable. The average percentages of nematocyst types in the tentacles nearest the mouth and in peripheral tentacles are given at week 0 and week 9.*

Nematocyst type	Tentacle type					
	Catch tentacles	Tentacles nearest the mouth			Peripheral feeding tentacles	
	Week 0	Week 0	Showing increased opacity At week 9	Not showing increased opacity At week 9	Week 0	Week 9
Spirocysts	0%	82%	0%	81%	82%	84%
Microbasic *b*-mastigophores	0	16	0	17	16	14
Microbasic amastigophores	1	2	2	2	2	2
Atrichs	40	0	39	0	0	0
Holotrichs	59	0	59	0	0	0

nine weeks of growth closely approach those found in fully formed catch tentacles in animals collected from the field (Table III).

Several tentacles in the first circle surrounding the mouth in a majority of individuals from the two clones developed into functional catch tentacles. The time of first appearance and the number of catch tentacles formed varied between clones and between individuals in the same clone. The transformation of feeding tentacles into catch tentacles, including the dramatic change in nematocyst types, was nearly completed in nine weeks. Catch tentacles were inflatable after three to four weeks of growth, at which time all nematocyst types were present. Numerous interclonal attacks occurred with severe injury inflicted. Studies on catch tentacle induction are continuing.

Discussion

Evidence presented in this study for *Metridium senile* and by Williams (1975) for *Haliplanella luciaea, Diadumene cincta, Cereus pedunculatus, Sagartia elegans,* and *S. troglodytes,* shows that in these species the catch tentacles function in inter- and intraspecific aggression and not in feeding, as had been previously assumed. Considering these findings, the term "catch tentacle", implying a food-gathering function, is misleading. On present evidence a more appropriate functional name would be "fighting tentacle", in contrast to the smaller tentacles whose primary function is feeding. If future research on the other species with similar tentacles shows also function in aggression, renaming catch tentacles should be considered.

The catch tentacles are labile structures in *M. senile,* occurring where nonclonemates are adjacent. This study demonstrated their induction from regular feeding tentacles in individuals lacking catch tentacles during interaction with nonclonemates over several weeks. This externally triggered induction involves the development of a new set of nematocysts and a system for their continued production, a corresponding change in morphology, and the emergence of the behavior appropriate for implementation of the aggressive structures. Some interaction is needed to maintain the induced structures and behavior, for Hand (1955) described catch tentacle regression in isolated individuals.

The aggression between nonclonemates of *Metridium senile* is analogous to that described for *Anthopleura elegantissima* by Francis (1973a), with interesting parallels. In both species tentacle tip contact with nonclonemates stimulates aggression. The development of structures used in aggression is greatest in individuals most likely involved in aggression. Francis (1976) found that individuals of *A. elegantissima* at inter-clonal borders had more and larger acroraghi (which function is aggression) than other clonemates.

The aggressive ability of each anemone species and each clone probably has a profound effect on the competition for space. Lang (1973) showed that interspecific aggressive interactions were critical in the development of community structure in scleractinian corals. Depending on the species' position in an invariable hierarchy of aggressiveness, aggression prevented a colony from being overgrown by another species, or enabled one species to overgrow others. Rogers (Hopkins Marine Station, unpublished report) demonstrated a hierarchy in aggressiveness among four clones of *Anthopleura elegantissima.* Where several species of anemones co-occur, aggression may be an important determinant in their distributions. This is suggested by Chao (Hopkins Marine Station, unpublished report), who showed that a hierarchy of aggressiveness exists among three species of anemones whose populations co-occur but do not intermingle at Monterey Wharf #2: *Metridium senile,* tested without catch tentacles, was most aggressive, followed by *Corynactis californica,* and finally by *Anthopleura elegantissima.*

Each anemone clone, consisting of genetically identical individuals, is comparable to an organism upon which natural selection is acting. The more space occupied by the clone, the greater the available area for food capture. This should allow the clone to expand more rapidly by asexual means, resulting in a larger number of sexually reproducing individuals. Thus, the aggressive ability of the clone, being critical in maintaining and expanding its area, would seem to be of substantial adaptive value.

My sincerest thanks go to Dr. Donald Abbott and Charles Baxter, who contributed immeasurably to every aspect of this study. Christopher Kitting kindly took the photographs on which field data were based and those documenting behavior. I appreciate the comments made upon the manuscript by Dr. James Case and Dr. Demorest Davenport. I also wish to thank Dr. Cadet Hand for his suggestion that I re-examine catch tentacle function in *Metridium senile.*

SUMMARY

1. The "catch tentacles" of *Metridium senile* are larger and more opaque than normal feeding tentacles, are capable of great enlargement, and possess a cnidom differing strikingly from that of the regular or feeding tentacles.

2. The catch tentacles are not used in food capture. Inflated or resting catch tentacles never accepted food in animals which were feeding with regular tentacles.

3. Genetically different clones of *M. senile* are often separated by narrow open corridors free of anemones. Specimens of *M. senile* bearing catch tentacles are found along the borders of these corridors, and in most situations where individuals of different clones are adjacent and within reach of one another.

4. Catch tentacles are used in both intra- and interspecific aggression. Prolonged and repeated feeding tentacle contact between nonclonemates and clonemates where the individuals have been isolated from contact with all other anemones for a period results in the expansion of catch tentacles. Catch tentacle expansion sometimes occurs without an apparent stimulus. Nematocyst discharge of the catch tentacle tip occurs only upon contact with a genetically different individual. After contact and discharge the catch tentacle breaks, leaving about 1 cm of the tip attached to the victim; necrosis at this site follows, and occasionally the victim dies.

5. Catch tentacle formation in individuals lacking them was observed in members of two clones which were confined in close quarters for nine weeks. Feeding tentacles closest to the mouth became enlarged and more opaque, lost their feeding nematocysts and developed nematocysts characteristic of catch tentacles. The transformation was largely completed in nine weeks.

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CALCIUM STORAGE AND DISTRIBUTION IN THE DIGESTIVE GLAND OF *BENSONIA MONTICOLA* (GASTROPODA: PULMONATA): A HISTOPHYSIOLOGICAL STUDY

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The pulmonate digestive gland is so well-mined an area of investigation that the reasons for another study on it seem to rest on shaky ground. The structure of this organ has been well known for over eighty years now; it consists of numerous tubules (acini) surrounding the intestine and separated from one another by interacinar spaces. The acini are constructed of an epithelium of various cell types, and a long, as yet unresolved, controversy has raged over whether there are three kinds or four. Even here, much is to be said in favor of the view of Abolins-Krogis (1970) that all types except calcium cells are different functional manifestations of one basic type. Whatever be the ultimate answer to that, it is undisputed that one of the cell types is the calcium cell. It is also uncontested that in addition to serving the function of digestion, this organ in gastropods serves for calcium storage as well (Manigault, 1939; Abolins-Krogis, 1961). An extensive body of literature (*e.g.*, Abolins-Krogis, 1968; Saleuddin, Miranda, Losada and Wilbur, 1970) attests that this organ also releases calcium and other substances in times of increased demand, such as during shell regeneration. The release of calcium is mediated by the breakdown of calcium spherites within the calcium cells (Abolins-Krogis, 1961). While the enormous contribution of this author to knowledge of these spherites is beyond question, much still remains unknown regarding them. Wilbur (1972, p. 108) states that "the spherites, consisting of both mineral and organic components, merit further attention as calcifying systems." It may be mentioned here that these spherites are also of interest by reason of being intracellular calcifying systems, in contrast to shell and bone.

The present communication attempts to elucidate the mechanism of calcium uptake, storage and distribution by this gland. In sum total the amount of calcium taken up by the mollusc is always greater than that lost in normal physiological processes such as shell formation and excretion, and also its level in the blood is maintained within narrow limits (Greenaway, 1971). This points to some sort of regulatory mechanism. Some exceedingly simple hypotheses have been put forth to account for the observed constancy of blood calcium, such as precipitation during periods of saturation in the blood and solubilization in times of depletion. However, matters may not be as simple as that, and the present work proposes a specialized cellular process that may be capable of finer regulation.

MATERIAL AND METHODS

Specimens of *Bensonia monticola* (Gastropoda: Pulmonata) were collected from the hills surrounding Solan (Himachal Pradesh, India) during the months of July-September, 1971–1973. The soil of the locality is not visibly calcareous. The shells were cracked open, and the digestive glands were extirpated and divided into small pieces not exceeding 3 mm in any dimension. These were placed in the required fixative (Zenker, alcoholic Bouin, neutral formalin, and absolute ethanol), dehydrated, infiltrated in paraffin wax and sectioned at 7 μ. The sections affixed to glass microslides were subjected to the following histological procedures: hematoxylin-eosin, iron hematoxylin, and Gomori's trichrome staining (Lillie, 1965). While many histochemical methods were applied, only the following are of relevance here, and were performed as detailed in Pearse (1961) unless stated otherwise: von Kossa method for "calcium"; the same, counter-stained with neutral red; Dahl's alizarin red S method for calcium; purpurin method for calcium (Gurr, 1962); alcian blue method for acid mucopolysaccharides (AMPS); the same, counterstained with neutral red; toluidine blue for AMPS and metachromasia in general; Bensley's permanent toluidine blue method (Gurr, 1962), and the periodic acid-Schiff (PAS) method for other carbohydrates. All tests for calcium were performed on material fixed in acid-free fluids (neutral formalin/absolute ethanol); furthermore, all calcium tests (and certain others, see Observations and Results) were accompanied by decalcification controls effected by immersion of the slides in 6% EDTA for 30 min or 5% HNO_3 for 10 min, followed by a distilled water wash. Photomicrography was done with an Asahi Pentax Spotmatic camera mounted upon a Carl Zeiss Jena research microscope.

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Observations and Results

The digestive gland of *Bensonia monticola* is a dark brown structure forming the greater mass of the visceral hump. The intestine excavates a convoluted pathway through this mass, being generally cut across more than once in a given section. The remainder of the space is occupied by the transected tubules (acini), which are constructed of an epithelium one cell in thickness. Most of the cells are distally vacuolated. The acini are separated by interacinar spaces which originate beneath the intestinal epithelium. A section of this gland, if stained for calcium, shows the peripheries of the acini brightly outlined with it (Fig. 1). At low magnification it may seem that these deposits lie in the interacinar spaces, but it is very clear at higher magnifications (Fig. 2) that they lie within the calcium cells. They are in the form of spherites, generally not more than five per cell. Each stains dense black with the von Kossa method and bright, fire-orange with the alizarin and purpurin methods. They are completely eliminated by decalcification of the sections prior to staining. The spherites occupy a basal or nearly basal position in the pyramidal calcium cell, which often terminates in a filiform process on the luminal side of the acinus. There is a large and more or less spherical nucleus with chromatin dots and a single nucleolus. The cytoplasm appears to be progressively less dense towards the luminal pole of the cell. The same illustration (Fig. 2) also shows digestive and excretory cells and the nuclei of the undifferentiated interstitial cells, which give rise to the other cell types. The interacinar space (which originates in the sub-intestinal region) is very clear.

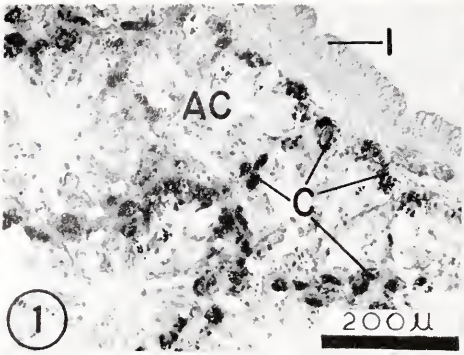
Following the localization of calcium spherites an attempt was made to ascertain whether any acid mucopolysaccharide(s) (AMPS) or other carbohydrate lay in association with them. It was thought reasonable to expect AMPS, but perplexingly, staining in alcian blue was either negative or so poor that localization could not be determined. The PAS reaction was also negative. However, one batch of slides was left inadvertently in alcian blue overnight, and it was discovered the next day that a great deal of staining had taken place. Since the normal staining time with alcian blue does not exceed 30 min, it was reasoned that something other than staining was occurring and that this could be decalcification, since alcian blue is made up in 3% acetic acid. Therefore, a fresh set of preparations was run through alcian blue after having been decalcified in EDTA and HNO_3. A remarkable change in the picture occurred, with staining time now being only 15 min (Fig. 3). The whole field was liberally sprinkled with alcianophilic spherules. These spherules were brightly reddish-metachromatic in toluidine blue. They may therefore be interpreted as being granules of AMPS. Closer examination reveals that the spherules are nothing but the organic centers of the calcium spherites left behind after decalcification (Fig. 4), because it is not possible to mistake a calcium cell for any other on account of its distinctive outline and the absence of distal vacuolation. Furthermore, the spherules occupy positions identical to those of the calcium spherites.

Thus the calcium cells are the sites of calcium storage in the digestive gland of *Bensonia*, and the storage is in the form of spherites built around AMPS spherules.

Additionally, there is one more structure of interest, which can be seen whenever sections are stained for calcium. This takes the form of rings which stain dense black in von Kossa's method. Upon close examination it may be confirmed that each ring encloses a blood vessel. This is especially clear when the ring is cut obliquely or longitudinally, and the blood vessel may then sometimes be seen to bifurcate or trifurcate. It may be ascertained that the rings and the branching blood vessels are the same objects in different orientations when one sees that they both contain the same kind of cells—the amoeboid blood corpuscles.

The structure of the rings was enigmatic because they were first discovered in von Kossa-stained sections. This method yields a dense and optically impenetrable black deposit through which no detail may be discerned. It was therefore first thought that the ring represents an amorphous calcium deposit in the form of an ensheathing tube around the blood vessel. The fact that this is not so can be seen when companion sections are stained by alizarin (Fig. 5) and purpurin methods. Here it may be ascertained that each ring is made up of distinct cells whose cytoplasm is choked with calcium granules, and that these cells surround the blood vessels completely. No nucleus was found. In this work these cells have been named the *calcium distributing cells.*

The reason for so naming them is as follows: under high magnifications it can be seen very distinctly that small granules of calcium (as carbonate) lie outside these cells in the basal (interacinar) regions and similar fine granules appear in the lumen of the blood vessels. The cytoplasm of these cells is already full of calcium (Fig. 6). The author has surmised that these cells take up calcium basally and extrude it through the thin and perhaps discontinuous walls of the blood vessels luminally, whence it is borne away by the bloodstream. There can be no confusion in this matter, because the lumen is also seen to contain blood corpuscles (Fig. 6) already mentioned. However, not every individual shows uptake or extrusion or both.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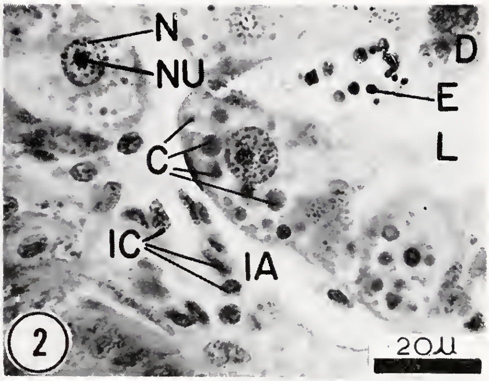
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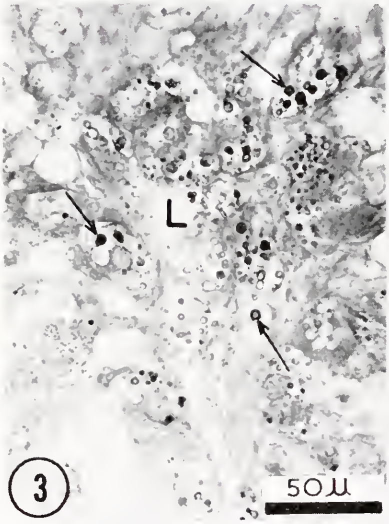
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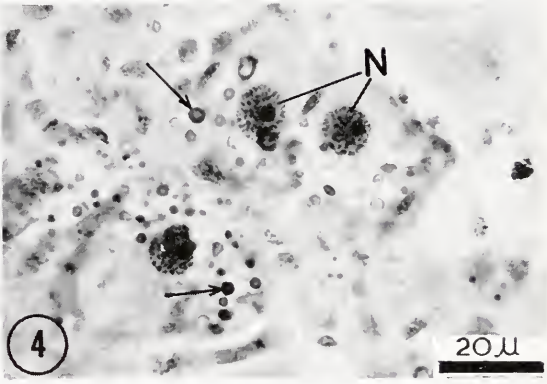
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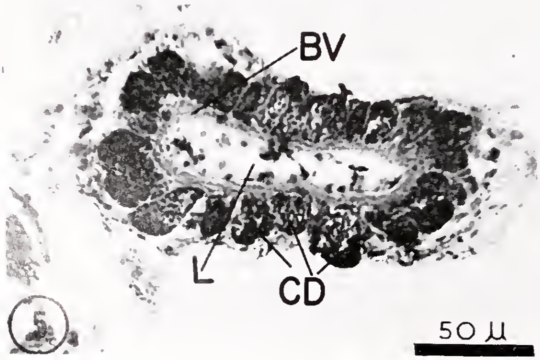
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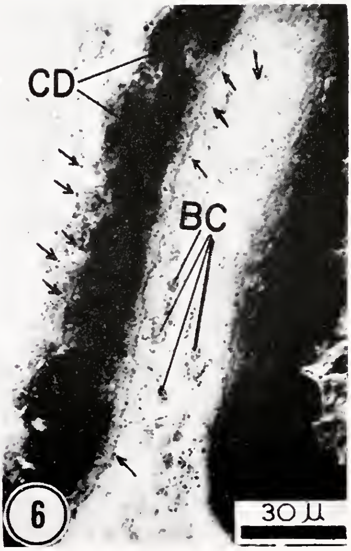
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Finally, although the calcium has been described as granular, it must be mentioned that this description applies to stained sections of fixed material only. There may not be any justification for assuming that transfer occurs in a granular form during life.

DISCUSSION

From the foregoing observations it will be evident that calcium can be localized histochemically in two sites: the calcium cells of the acini and the calcium distributing cells. When food material is present within the lumen of the sectioned intestine, it can also be seen to yield positive reactions for calcium. Though the intestinal epithelium does not stain for calcium, it is easy to surmise that calcium passes through it from the lumen (possibly very rapidly but in histochemically undetectable quantities) into the interacinar spaces. From here it is fairly easy for calcium cells to acquire it through their basal poles.

It is very well known (see Abolins-Krogis, 1961) that these calcium cells do give up their stored calcium in times of need by breakdown of spherites and that the spherites contain AMPS and other organic matter.

Therefore at first sight the calcium distributing cells may seem an organic redundancy, because the functions of storage and distribution are both handled by the calcium cells of the acini. It may also initially seem reasonable that calcium distributing cells are not necessary because once calcium enters the interacinar spaces, it is already in the hemocoel and hence in the bloodstream; any cell serving merely to inject calcium into the bloodstream would appear superfluous.

It may be mentioned here that calcium distributing cells are not being reported for the first time. Greenaway (1971) quotes Carriker (1946) as having found "cells containing calcium deposits lining the outer walls of arteries and capillaries in *Limnaea stagnalis appressa*". This meager information is the only one encountered by the author regarding these cells in published literature, but he is informed (U.

FIGURE 1. Peripheries of acini (AC) demarcated by abundant calcium deposits (C). The intestine (I) is visible at upper right; neutral formalin/alizarin red S.

FIGURE 2. Small area from two adjacent acini showing calcium cells and others; the former contain calcium spherites (C) and spherical nuclei (N) with prominent nucleoli (NU). Also seen are a digestive (D) and excretory (E) cell. L is the lumen of acinus at right, while IA marks the well-preserved interacinar space between left and right acini. Nuclei of several interstitial cells (IC) also visible; neutral formalin/von Kossa-neutral red.

FIGURE 3. Staining with alcian blue following decalcification in EDTA. Numerous AMPS spherules (arrows) stain. The acinus is sectioned longitudinally. L marks its lumen; neutral formalin/alcian blue after decalcification in EDTA.

FIGURE 4. Three calcium cells after decalcification, stained to show AMPS spherules. Note similarity of their nuclei (N) with those of Figure 2 and that the spherules occupy a position identical to that of calcium spherites in Figure 2; neutral formalin/alcian blue-neutral red after decalcification in EDTA.

FIGURE 5. Transversely sectioned blood vessel in the digestive gland. Note calcium distributing cells (CD) outside the connective tissue wall of the blood vessel (BV) and blood corpuscles in the lumen (L); neutral formalin/alizarin red S.

FIGURE 6. Longitudinally-sectioned blood vessel showing intense staining in calcium distributing cells (CD), which are taking up (small arrows at left) particulate calcium and also releasing it into the lumen (arrows in lumen). The nuclei of several blood corpuscles are very clear (BC); neutral formalin/von Kossa-neutral red.

Kanwar, Department of Zoology, Panjab University, personal communication) that rings have also been seen in the digestive gland of another land pulmonate (*Euplecta indica*) from Chandigarh. This would mean that *Bensonia* is only the third pulmonate in which these have been observed. However, no one seems to have speculated upon their function.

The present author believes that the following may be a possible explanation for the calcium pathway in the digestive gland of these pulmonates: calcium is acquired *per os* and enters the interacinar spaces *via* the intestinal epithelium. These are hemocoelic spaces (analogous, roughly, to venous sinusoids), and so the calcium is now in the vascular system. However, the spaces are extensive and dilated in many parts of the body. The movement of hemocoelic fluid within them is sluggish and sufficiently slow to permit the transport of this newly-acquired calcium to the calcium cells in the acini, which take up as much as is required. The interacinar spaces, it is seen, also extend to the rings surrounding the blood vessels, and the surplus calcium is taken up by the calcium distributing cells. In contrast to the situation in the calcium cells of the acini, the calcium here is in a rapidly mobilizable condition, since no spherite breakdown is required and there is no binding organic matrix. The first demand for increased calcium in the blood is met by these calcium distributing cells, which directly inject granular calcium carbonate into the fast-moving blood within the small-bored blood vessels (roughly analogous to arteries and often named as such) which they surround. A greater and more sustained demand (*e.g.*, during shell repair) can be met only slowly by the breakown of calcium spherites within calcium cells of the acini. The calcium from these disintegrated spherites would possibly either slowly percolate through the interacinar spaces and thus fulfill long-term needs or be transferred to the calcium cells for dispersal from there. Besides, the AMPS centers of these spherites would cause firm binding with calcium and permit only slow dissociation. This is indicated by their total inaccessibility to proper staining by alcian blue and their orthochromasia in toluidine blue unless the section is thoroughly decalcified, when their reactive anionic groups are unmasked. The same phenomenon has been seen in reverse order in the pulmonate *Euplecta indica* (Kapur and Sen Gupta, 1970), where the metachromasia of AMPS decreases with progressively greater degrees of calcification, thus indicating more and more binding of Ca^{++} by AMPS. The capability of AMPS to bind metallic ions is well documented (Rao and Goldberg, 1954; Simkiss and Tyler, 1958); also, Horiguchi (1956) and Horiguchi and Miyake (1954) have shown that AMPS extracted from the tissues of *Hyriopsis* and *Pteria* always contain a calcium residue. AMPS are almost universal accompaniments of calcifying systems, being found in such diverse situations as bone, dentine, and enamel in vertebrates, in the egg shells of birds, and in calcified structures of echinoderms, crustaceans, molluscs, sponges, protozoans and even bacteria among the nonvertebrates (for a comprehensive bibliography see Kobayashi, 1971). On the other hand, no such binding occurs in the calcium distributing cells, which present identical pictures in methods for AMPS with or without prior decalcification. In other words, calcium in the calcium distributing cells is highly labile compared to that in the calcium cells of the acini.

A precedent exists in support of the belief that there is a slow- and fast-moving system in the digestive gland. Discussing the turnover of exchangeable tissue calcium in *Limnaea stagnalis*, Greenaway (1971) stated that exchange of digestive gland calcium appeared to comprise a slow and fast component. He admitted the possibility that the slow component may not represent exchange between blood and digestive gland calcium, but *deposition* of further calcium in the cells of the tissue. This would correspond to the buildup of spherites in the calcium cells in the present instance.

Therefore, it is possible that a mechanism resides in the pulmonate digestive gland for the fine control of blood calcium, and that this has been overlooked for thirty years, since the time of Carriker (1946). Its existence can be proved by the use of autoradiography, but that is beyond the reach of facilities available in this department. This being so, it must be cautioned that the theories regarding calcium uptake and extrusion are based on the appearance of stained sections. There is as yet no experimental evidence in their support; these statements are hypothetical and must be treated as such.

Also, stimuli responsible for the "triggering" of calcium release and the control of its deposition must at this time remain conjectural.

The author is grateful to Dr. G. P. Sharma, Head, Department of Zoology, Panjab University, Chandigarh, for having provided him with all necessary laboratory facilities, and more so for encouraging him and sustaining his will in times of difficulty.

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The author places on record his appreciation of the kindness of Dr. Mrs. Venna Suri (née Sawhney), who went out of her way to secure specimens of *Bensonia* from Solan.

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SUMMARY

The interrelationship of the digestive gland mass and the intestine in a land pulmonate, *Bensonia monticola*, is described. Calcium is found in two principal locations, the calcium cells in the acini and in the calcium distributing cells surrounding the blood vessels. The latter have been barely mentioned in previous literature. The calcium cells in acini contain calcium bound to acid mucopolysaccharides in the form of spherites, whereas the calcium in the calcium distributing cells is unbound, granular and labile. It appears that the latter kind of cell extrudes calcium directly into the blood vessels. The author submits that these two cell types may be responsible respectively for fulfilling slow, long-term needs and rapid, immediate demands, and that together they constitute a fine system of blood calcium regulation that has been overlooked for nearly thirty years now.

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MIXED FUNCTION OXYGENASE ACTIVITY IN BLUE CRAB, *CALLINECTES SAPIDUS:* TISSUE DISTRIBUTION AND CORRELATION WITH CHANGES DURING MOLTING AND DEVELOPMENT

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A group of microsomal enzymes, referred to as mixed function oxygenases, are responsible for the metabolic modification of many foreign compounds in animals. Due to the introduction of industrial wastes and fossil fuels into the ocean, the fate and effects of foreign compounds in marine animals are of interest. Uptake and discharge of petroleum hydrocarbons at nontoxic levels have been examined in several marine species including fish (Lee, Sauerheber and Dobbs, 1972b), crabs (Corner, Kilvington and O'Hara, 1973; Lee, Ryan and Neuhauser, 1976), shrimp (Anderson, Neff, Cox, Tatem and Hightower, 1974), mussels (Lee, Sauerheber and Benson, 1972a), clams (Neff and Anderson, 1975) and oysters (Stegeman and Teal, 1973; Stegeman, 1974). Hydrocarbons may be eliminated from marine animals either unmodified or as polar metabolites (Stegeman and Teal, 1973; Corner *et al.*, 1973). Arylhydrocarbon hydroxylase, a mixed function oxygenase, is involved in the hydroxylation of the aromatic ring, an early step in the metabolism of aromatic hydrocarbons. This enzyme has been characterized in mammals (Conney, Miller and Miller, 1957; Nebert and Gelboin, 1968; Gelboin, 1972; Owens and Nebert, 1976; Atlas and Nebert, 1976), fish liver (Pedersen, Herschberger and Juchau, 1974; Payne and Penrose, 1975; Philpot, James and Bend, 1976) and is present in green gland of fresh water crayfish (Khan, Coello, Khan and Pinto, 1972) and blue crab (Lee, Furlong and Singer, 1977). The occurrence of enzyme activity in several tissues of the blue crab, *Callinectes sapidus*, with varying levels of activity dependent on both sex and stage of development, is reported in this study.

MATERIALS AND METHODS

Blue crabs, *Callinectes sapidus*, were collected by trawling or trapping in estuaries of coastal Georgia (U.S.A.) between May and September when the median water temperature is 28° C. The crabs were maintained in tanks receiving flowing filtered sea water of 20‰ salinity (White, Stickney, Miller and Knight, 1973) and were segregated according to sex and size. Those specimens in the final stages prior to ecdysis or for special treatment (diet or eyestalk removal) were placed in individual aerated seawater aquaria. All crabs were fed three times weekly with frozen shrimp.

Internal tissues were located, identified and dissected using references to the general biology of the blue crab (Cronin, 1947; Pyle and Cronin, 1950; Waterman and Chace, 1960; Passano, 1960; Bollenbacher, Flechner and O'Connor, 1972; Carlisle and Connick, 1973). Stages of the molting cycle were classified using the guidelines summarized by Passano (1960).

Crude homogenates were prepared from dissected tissues in 0.15 M KCl buffered with 0.05 M Tris, pH 7.4, using a Potter-Elvehjem homogenizer. Cell debris and nuclei were removed by centrifugation at 700 × g for 10 minutes at 4° C. Supernatants were maintained at 4° C until assay of enzyme activity.

Arylhydrocarbon hydroxylase was assayed by the method of Wattenberg, Leong and Strand (1962), with the modifications described by Nebert and Gelboin (1968). The assay mixture contained 0.6 μmoles NADPH (Sigma Chemical Co.), 3 μmoles $MgCl_2$, 0.01 μmoles benzo(a)pyrene (Sigma Chemical Co.) and crude tissue homogenates (0–4 mg protein) in a total volume of one ml. The mixture was incubated at 28° C for 30 minutes and stopped by the addition of 1 ml cold acetone and 3 ml hexane. Two ml portions of the resulting organic phase were extracted with 4 ml normal NaOH and fluorescence of products recorded with activation of 396 nm and emission at 522 nm (Turner Model 430). Assays were done in triplicate with a blank containing homogenate boiled for 30 seconds prior to addition of substrates. The assay was determined to be linear with both time and protein under these conditions in green gland homogenates. One unit of enzyme activity was defined as the fluorescence produced in a 60 minute incubation at 28° C equivalent to the fluorescence of 1×10^{-12} moles of 3-hydroxybenzo(a)pyrene.

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as the standard.

RESULTS

Large male crabs of 14 to 17 cm in carapace width were judged mature. Specific activities of crude homogenates were determined in ten tissues (Table IA) as outlined in methods. Activity was detected in only nine tissues, the cardiac muscle apparently lacking activity. In blood, gill, reproductive tissues, eyestalk, and hepatopancreas, median values were less than 150 units activity per mg protein. Higher activity was seen in green gland homogenates (450 ± 270 units per mg protein). However, the highest activity in the male was found in the pyloric stomach which had 1400 ± 170 units per mg protein.

Mature female crabs were easily differentiated by the broadened abdomen (apron) on the ventral side of the body. Upon internal inspection, only mature females with developed ovaries were used to determine specific activities (Table IB). Specific activities in blood, gill, reproductive tissues, eyestalk, cardiac muscle, and hepatopancreas were about the same as in the male. The enzyme activity of pyloric stomach was slightly higher than that found in males, and enzyme levels of the female green gland were almost two orders of magnitude higher than found in the males.

Female specimens showed no decrease in green gland activity one day after removal of eyestalks. However, females which survived four and six days after eyestalk removal showed a decrease in green gland activity. After six days, the activity was approximately one half of control activity. The green glands are located near the base of the eyestalks and may be affected by a hormone secreting gland (designated x-gland) located within the eyestalk.

Growth in crustaceans requires a series of molts. In the female blue crab there are approximately 20 postlarval molts prior to maturity (Von Engel, 1958), with each molt resulting in an increase in carapace width of about one third. The final

TABLE I

Specific activities of arylhydrocarbon hydroxylase in various tissues of blue crabs. One unit of enzyme activity equals the fluorescence equivalent to 1 × 10^{-12} moles of 3-hydroxybenzo(a)pyrene produced in a 60 minute incubation of 28° C. Specific activity values are the mean ± standard deviation with the number of assays given in parentheses.

Tissue	Total activity (units)	Specific activity (units/mg protein)	
A. Mature Males			
green gland	950	450 ± 270	(8)
hepatopancreas	560	10 ± 12	(13)
testes	1,300	150 ± 110	(13)
median vas deferens	300	18 ± 13	(3)
proximal vas deferens	1,500	15 ± 15	(3)
gill	6,900	140 ± 80	(12)
blood	5,000	3.1 ± 1.1	(2)
pyloric stomach	20,000	1,400 ± 170	(4)
eyestalk	43	140 ± 26	(3)
heart	–0–	–0–	(1)
B. Mature Females			
green gland	109,000	34,000 ± 2,700	(7)
hepatopancreas	1,400	9.4 ± 6.5	(6)
ovary	170	7.7 ± 7.6	(4)
seminal receptacle	800	97 ± 91	(5)
gill	1,900	140 ± 124	(3)
blood	3,000	2.6	(1)
pyloric stomach	49,000	2,500 ± 910	(6)
eyestalk	90	87	(1)
heart	500	21	(1)

molt produces a mature female ready for breeding. Upon reaching maturity, evidence indicates that the female does not molt successfully again even though she may live up to three years. Arylhydrocarbon hydroxylase in green glands was measured in two general classes of immature female blue crabs. The first was judged to be in cycles prior to the final maturing molt. Enzyme activities of this group were compared according to carapace width. Crabs, 5 to 5.5 cm in size, molt to produce individuals of about 7 cm which, in turn, molt to produce 9 cm specimens. When plotted (Fig. 1), seven immature female crabs from these three molt cycles gave a linear correlation between green gland activity and carapace width ranging from 3400 to 29,000 units per mg protein. None of the crabs showed evidence, either external or internal, of being near ecdysis.

The second class of immature females was in the final molt cycle but not yet in the enforced fast which occurs three to seven days prior to ecdysis. The intermolt prior to this cycle extends up to seven months (Truitt, 1939). Specimens in this group were difficult to judge externally and internally, and enzyme levels of arylhydrocarbon hydroxylase varied greatly. Five specimens of the same carapace size (±0.5 cm) were graded stages 1 to 5 as the color of the apron darkened. Stages 1 through 4 were nonfasting and did not have molting rings on the walking legs. Stage 5 had the darkest apron and was nonfasting but did have pink molting rings

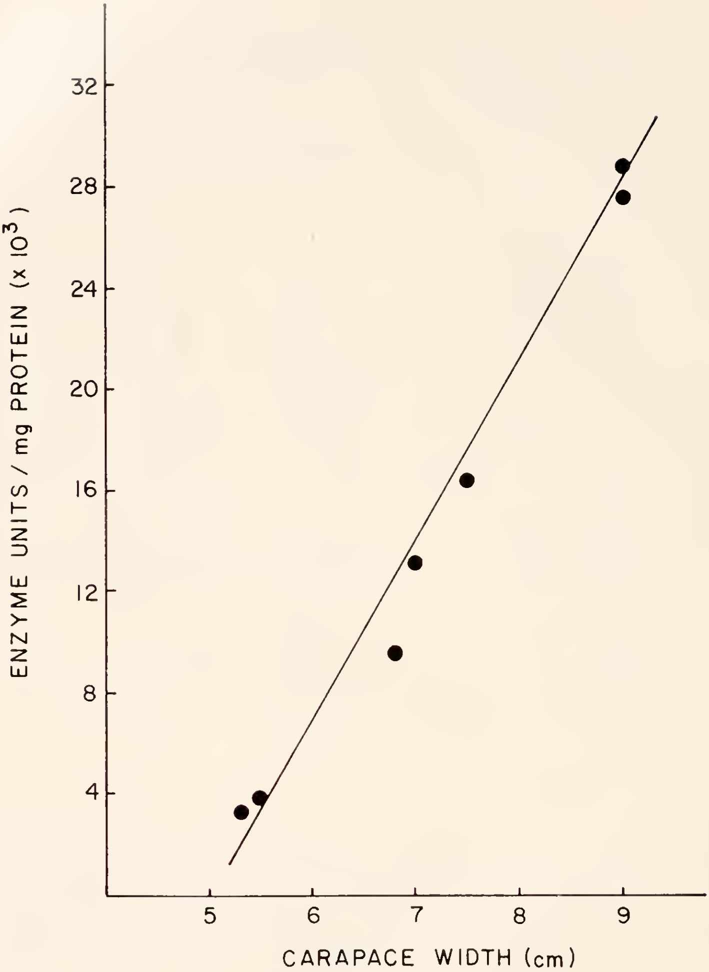

FIGURE 1. Increase in arylhydrocarbon hydroxylase activity in green gland of immature female blue crabs. Specimens chosen were intermolt from three different molt cycles prior to the final molt for maturation.

clearly visible on the walking legs. In general, as the color of the immature apron darkened, the enzyme level of green gland increased from 4,000 units/mg protein in stage one to 31,000 units/mg protein in stage four. Stage five, which was entering the later stages of proecdysis, had a green gland activity of 12,000 units/mg protein. Two females were examined which were in a fasting state which immediately precedes shedding (Table II). Both had less than one-tenth normal adult enzyme activity. Females taken during the first four hours after ecdysis showed enzyme activity levels of 7800 ± 4000 units per mg protein. Hardening of the new shell begins four or five hours after shedding and continues for about two days. Individuals which were ten hours and twenty hours postecdysis showed activities of 24,000 and 26,000 enzyme units per mg protein, respectively. After 60 hours, the enzyme had reached levels of 35,000 enzyme units per mg protein, well within the range of activity found in mature adults. Other tissues assayed included hepatopancreas, gill, and stomach. Excluding green gland, no significant variation from adult levels of arylhydrocarbon hydroxylase was found in tissues of female immature crabs.

One small *Callinectes sapidus* male (10.5 cm carapace width) was found, which upon internal inspection, was judged immature. Green gland activity measured 630 units per mg protein. This is within the range of the observed values in mature males. Other tissues measured were not significantly different in arylhydrocarbon hydroxylase from that found in mature males.

Previous reports have not noted significant activity in stomach tissue as was seen here in both mature male and female blue crabs (Table I). A large male crab was carefully dissected and fluid drawn by needle from the unruptured pyloric stomach. The stomach was then removed, its contents discarded, and the tissue carefully washed with buffer. The juice from the stomach had no detectable activity and when the fluid was added to the *in vitro* assay of stomach homogenate, no inhibition was observed. The effect of starvation on enzyme activity of the stomach was examined. Mature females were starved up to six days and enzyme activity determined daily. During this time no decrease in activity occurred.

DISCUSSION

In this study, very low levels of the mixed function oxygenase, arylhydrocarbon hydroxylase, were found in *in vitro* assay of all tissues examined of *Callinectes sapidus* except the stomach and green gland. Recently, several tissues of the fiddler crab were surveyed for mixed function oxygenase using the aldrin epoxidation reaction (Burns, 1976). In agreement with our observations, the green gland had the highest activity, while in hepatopancreas much lower activities were noted. In contrast, the *in vivo* data of Lee *et al.* (1976) concluded that the hepatopancreas is the site of hydrocarbon metabolism in the blue crab. Pohl, Bend, Guarino and Fouts (1974) postulated that digestive fluids produced in the hepatopancreas inhibited the *in vitro* assay of the enzyme.

In stomach tissue, significant levels of arylhydrocarbon hydroxylase were found.

TABLE II

Arylhydrocarbon hydroxylase in the female green gland just prior and after the final molt. Specific activity values are the mean ± standard deviation with the number of assays given in parentheses.

Stage	Time	Specific activity (units/mg protein)
Proecdysis		
fasting	3–7 days	2,000 ± 1,000 (2)
Postecdysis		
soft shell	0–4 hours	7,800 ± 4,400 (3)
paper shell	10–20 hours	25,000 ± 1,300 (2)
hard shell	2–14 days	35,000 ± 1,000 (2)

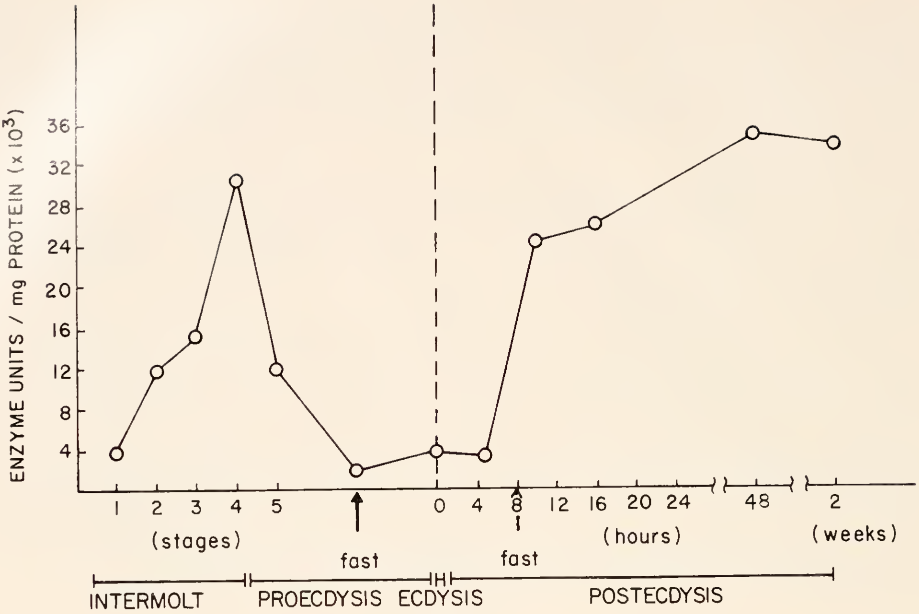

FIGURE 2. Fluctuation of arylhydrocarbon hydroxylase during molting in the green gland of the female blue crab. Intermolt stages were arbitrarily judged by darkening abdomen color. Proecdysis was judged by appearance of molting rings followed by a fast beginning 3–7 days prior to ecdysis. Postecdysis measured from moment when newly molted crab was free of old carapace.

This was unexpected, since the stomach is a chitinous structure and is shed at each molt. The activity was not due to any digestive fluid or parasites inside the stomach but rather was present in the washed tissue. In the male, the stomach accounted for most of the total activity. In experiments where crabs were fed a single dose of radiolabeled hydrocarbons, significant amounts of metabolites produced after one day were found in the stomach, whereas the hepatopancreas and blood contained more metabolites at longer time periods (Lee *et al.*, 1976). Additional experiments were not reported to remove the possibility that metabolites found in the stomach during *in vivo* studies were perhaps due to bacteria or other parasites. It would appear that the stomach, though not the primary site of metabolism, participates in the metabolism of aromatic hydrocarbons which are taken up from food.

The specific activity of arylhydrocarbon hydroxylase in green gland of egg bearing females was the highest found. Careful dissection of tissue eliminated possible contamination of green gland homogenates by tissue of the x-gland from within the eyestalk or by the y-organ (mandibular organ). However, a gland reported by Carlisle and Connick (1973) in the antennary segment of crayfish was not excluded from the green gland homogenates. In fact, some confusion exists as to whether or not these are indeed different tissues. Arylhydrocarbon hydroxylase activity in green gland increased in females as they progressed through the postlarval molts prior to maturity. Molting occurs during the growth of all arthropods. In insects, microsomal epoxidation activity fluctuates during development (Perry and Buckner, 1970; Yu and Terriere, 1971). Using data presented in the text and Table II, a summary diagram of arylhydrocarbon hydroxylase changes in crabs during development can be produced (Fig. 2) which appears quite similar to the changes in microsomal epoxidation activity in insects during their molting cycles (Wilkinson and Brattsten, 1972). In the female blue crab, molting rings became visible on the walking legs as the time of ecdysis approached, followed by an enforced fast. The enzyme levels which increased during intermolt began to fall and at ecdysis were at a very low level. Following ecdysis, the enzyme levels again rose as the newly molted crab proceeded through the soft and semisoft (paper) stages to a hardened exoskeleton. The crab did not begin to feed until the shell was completely hardened. Though males were not examined in this manner, it could be expected that enzyme levels would change in a similar pattern during molting. However, since the intermolt enzyme levels of males are much lower than females, the variation would not be as marked.

With live crabs, green glands showed no build up of any radiolabeled hydrocarbon and the presence of only polar metabolites (Lee *et al.*, 1976) as would be expected of an organ with mainly excretory functions. Why, then, is enzyme activity so high in a tissue with only known excretory functions and which in previous experiments has not been shown of *in vivo* importance? Fluctuations in enzyme activity during the molt cycles suggested a hormonal influence. This was further supported by the decrease in activity of green gland soon after removal of the eyestalks, which eliminated a supply of hormones produced by the x-glands.

Speculation as to the answer to the above question without further investigation is risky; however, green gland levels of arylhydrocarbon hydroxylase and molting hormones can be correlated in the following manner. Molting in crustaceans is controlled by a group of steroid hormones called ecdysones produced by the y-organs (Passano, 1960; Goad, 1976). During intermolt, a molt inhibiting hormone produced by the x-gland is present which suppresses the y-organ (Goad, 1976). Ecdysones were measured in *Callinectes sapidus* during three stages of the molt (Faux, Horn, Middleton, Fales and Lowe, 1969). The hormones were lowest in intermolt, higher in the proecdysis and highest just after ecdysis when the crab was soft. Arylhydrocarbon hydroxylase levels were inversely related to the ecdysones, were highest in intermolt, falling during proecdysis, and were lowest just after the molt when the crab was soft. Immediately following ecdysis, levels of arylhydrocarbon hydroxylase increased rapidly when the levels of ecdysones undergo an opposing decrease.

In addition to modification of foreign compounds, the mixed function oxygenases also function in the metabolism of bile acids, fatty acids and steroid hormones (Conney and Klutch, 1963; Greim, Trulzsch, Czygan, Hutterer, Schnaffner, Popper, Copper and Rosenthal, 1973; Czygan, Greim, Trulzsch, Rudick, Hutterer, Schaffner, Popper, Rosenthal and Cooper, 1974). In hepatic tissues, steroids are the likely natural substrates of the mixed function oxidases (Conney, 1967), and the interactions of chlorinated hydrocarbons with steroid hormones has been recently reviewed (Kupfer and Bulger, 1976). The green gland as an excretory organ monitors the blood and controls ion levels of the hemolymph (Robertson, 1960). It appears from our results that the green gland may also function in metabolism of steroid hormones. Crustecdysone, a steroid molting hormone, is a 20-hydroxy derivative of a cholesterol-like precursor which has been found by Carlisle and Connick (1973) to be present only in a gland of the antennary segment of crayfish. Conversion of dietary derived cholesterol to crustecdysone would require a 20-cholesterol hydroxylase activity. The enzyme activity of green gland attributed to arylhydrocarbon hydroxylase may be a similar steroid hydroxylase.

The biological effects of foreign compounds on marine life are not well understood. In addition to acute toxicity, reduced growth and reproduction pose a threat to the survival of marine organisms. If mixed function oxygenases contribute to the control of molting hormone levels, then the presence of aromatic hydrocarbons acting as substrates for these enzymes could alter the rate at which the crab passes through the early molts. Several studies with chlorinated hydrocarbons, a second group of foreign compounds, have shown that juvenile decapods are more sensitive than adults (Epifanio, 1971; Nimmo, Blackman, Wilson and Forester, 1971). Increasing concentrations of methoxychlor prolonged larval development to the first juvenile stage of the blue crab, *Callinectes sapidus,* the mud crab, *Rhithropanopeus harrisii* (Bookhout, Costlow and Monroe, 1976), and the dungeness crab, *Cancer magister* (Armstrong, Buchanan, Mallon, Caldwell and Millemann, 1976).

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SUMMARY

We have presented results of assay of various internal tissues of blue crabs, *Callinectes sapidus,* finding high activity of arylhydrocarbon hydroxylase in the stomach and green gland. Also we have noted that the green gland activity of this enzyme varies at different stages of maturity and molt cycle in a manner similar to insects. We have speculated as to why an excretory organ should have such high activity and undergo such fluctuations in activity.

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STUDIES ON TETRAPHYLLIDEAN AND TETRARHYNCHIDEAN METACESTODES FROM SQUIDS TAKEN ON THE NEW ENGLAND COAST[1]

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Early investigators, Verrill, Baird, Leidy, and others, studied the marine fauna of the Atlantic coast of North America. They reported developmental stages of tapeworms in various invertebrates and in fishes. Morphological agreement of plerocercoids from the several host-species presaged taxonomic relationship but posed an enigma: the presence of similar larvae in such diverse host-species. No information on life-histories of marine cestodes was available, and determination of life-cycles under experimentally-controlled conditions was far in the future. Professor Verrill of Yale University had published his monograph on the vertebrate animals of Vineyard Sound and in 1881 directed Edwin Linton, a young graduate student, in the study of helminthic parasites of fishes. It was the first systematic investigation of helminthic parasites in America. The study was continued for the next forty years, chiefly in the laboratories of the U.S. Fish Commission at Woods Hole, Massachusetts; Beaufort, North Carolina; Flatts, Bermuda; and Tortugas, Florida.

Now, a century later, information on these cestodes is still meager, and no life-history has been experimentally demonstrated. But it is clear that the worms have three larval stages and three successive hosts in the life-cycle; that they belong to the two orders, Tetraphyllidea and Tetrarhynchidea, and that they become mature in the spiral valve of selachian fishes. The collecting department of the Marine Biological Laboratory provides large numbers of squid, *Loligo pealeii*, for experimental work on neurophysiological problems and this material afforded the opportunity to study the cestode larvae harbored by them. The morphology and taxonomic relations of the plerocercoids are presented in this report.

Larval cestodes encysted in marine fishes have been known since O. F. Mueller (1787) erected a genus, *Scolex*, for three unnamed species. According to Stiles and Hassall (1912), *Scolex* was not proposed as a larval genus and *Scolex pleuronectis* Mueller, 1788 was accepted as type. *Scolex lophi* Gmelin, 1790; *Scolex cyclopteri* Fabricius, 1794; *Scolex marinus* Fabricius, 1794; and *Scolex pleuronectis platessa* Viborg, 1795 were named before 1800. All were found in fishes. The doubtful validity of these variable and inadequately described species led Rudolphi to suspect that all were members of a single species, which he (1819) designated as *Scolex polymorphus* Rudolphi, 1819. Later observations disclosed that these worms are juvenile forms of strobilate cestodes and that the name, *Scolex,* referred to a group of larval stages and corresponded to the names, *Redia* and *Cercaria*, that were proposed initially as generic designations. These parasites are often described as larvae, but actually they are juvenile stages of tapeworms that com-

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plete their development and become sexually mature in the digestive tracts of elasmobranch fishes, sharks and rays. For these post-larval stages, Wardle and McLeod (1952) proposed the term, metacestode, to conform with the designation, metacercaria, for the corresponding stage in the life-cycles of digenetic trematodes. Metacestodes have been reported from various marine invertebrates as well as fishes, and it appears that fishes acquire the parasites by ingesting invertebrates. In a series of papers, Dollfus (1923, 1924, 1929, 1931, 1964, 1967, 1974) listed the cestodes of the plankton and the marine invertebrates. No complete life-cycle has been proved experimentally, but the pattern of development has been constructed from isolated observations on many species. Observations on development of certain species have made it possible to assign them tentatively to families in the orders Tetraphyllidea and Tetrarhynchidea.

The taxonomy and nomenclature of these cestodes is controversial. The name Tetraphyllidea was proposed by Carus (1863) to latinize the names, Tétraphyllidés van Beneden, 1849 and Tétraphyllés van Beneden, 1850, for the cestodes in which the scolex bears leaflike or earlike outgrowths. The cestodes in which the scolex bears armed, protrusible and retractile tentacles, also called proboscides, were listed as Tétrarhynques by Blainville (1828). The name Tétrarhynchidéa was proposed to replace Tétrarhynchidés Gervais and van Beneden, 1859 and the name was validated by Claus (1891) according to Olsson (1893). For this second group of cestodes, Diesing (1863) proposed the name Trypanorhyncha, and it has been widely adopted. Braun (1900) in *Bronn's Klassen und Ordnungen,* adopted the name Trypanorhyncha, while Fuhrmann (1930) in the *Kükenthal und Krumbach, Handbuch der Zoologie,* recognized the order Tetrarhynchidea. In the *Faune de France,* Joyeux and Baer (1936) accepted the order Tetrarhynchidea Olsson, 1893 and the same authors (1961) in the *Traité de Zoologie* attributed the order to Claus, after Olsson, with no date. There was no mention of the name Trypanorhyncha Diesing, 1863. Dollfus (1942) adopted "l'ordre des Tétrarhynques (Trypanorhyncha Diesing, 1863)" while Wardle and McLeod (1952) and Yamaguti (1959) recognized the order Trypanorhyncha Diesing, 1863, with no mention of Tétrarhynchidea. Since the group is based on the several larval forms described by Redi (1684) and included in the genus *Tetrarhynchus* Rudolphi, 1809, the name Tetrarhynchidea appears more appropriate. *The Rules of Zoological Nomenclature* do not pertain to suprafamilial names, and each author may decide which names to accept. In the proceedings of a symposium held under the auspices of the American Association for the Advancement of Science on systematics of cestodes, Marietta Voge (1969) recognized six orders, all with the ending, *idea,* including the Tetrarhynchidea.

The helminthic parasites of marine fishes of the Atlantic coast of North America were studied by Edwin Linton for a period of more than fifty years. He (1897a) described and figured tetraphyllidean, and tetrarhynchidean larvae from fishes and squids. Some of the material he had collected at Woods Hole, Massachusetts, and the rest consisted of a collection from the U.S. National Museum. Many of the figures are deficient and meaningless, whereas others may be identified with considerable assurance. On Plate II, figures 1 to 9 depict specimens from *Illex illecebrosus* that were designated *Phyllobothrium loliginis* (Leidy, 1887) Linton, 1897. Other metacestodes from the same host-species, shown in figures 10 and 11, can be identified as *Dinobothrium septaria* (van Beneden, 1889) Braun, 1900. Figures 12 to 16, listed as *Rhynchobothrium* larvae from different fishes, suggest *Lacistorhynchus tenue* (van Beneden, 1858) Yamaguti, 1959. Figures 10 and 11, on Plate III and figures 3 to 8 on Plate IV are almost certainly of *L. tenue*. Indeed, Linton assigned the specimens to *Rhynchobothrium heterospine Linton*, 1890, now recognized as a synonym of *L. tenue*. Figures 9 to 12 on Plate IV were listed as *Rhynchobothrium imparispine* Linton, 1890, probably a synonym of *Grillotia erinaceus* (van Beneden, 1858) Yamaguti 1959, a species closely related to *L. tenue*, whose life-cycle was studied by Ruszkowski (1934). Figure 13 and 14 on Plate V were described as *Rhynchobothrium speciosum* new species; it was named type of a new genus, *Callotetrarhynchus*, by Pintner, (1931) and suppressed by Yamaguti (1959) as a synonym of *Tetrarhynchus gracilis* Rudolphi, 1819. Figures 1 to 5 on Plate VI were made from a single encysted specimen, designated *Otobothrium dipsacum* n. sp. The genus *Otobothrium* had been erected by Linton (1890) with *Otobothrium crenacolle* n. sp., a larva from various marine fishes, as type species. Dollfus (1942) included *O. dipsacum* in a new subgenus, *Pseudotobothrium*, which differs from *Otobothrium* in the length of the tentacular bulbs and the number of rows of large and small hooks on the tentacles. Yamaguti (1959) suppressed *Otobothrium insigne* Linton, 1905, as a synonym of *O. dipsacum*.

The biology and bionomics of the tetraphyllidean and tetrarhynchidean cestodes are known from fragmentary observations and from results of experimental studies on early larval stages. The adult stages of these cestodes live in the spiral valve of elasmobranch fishes, and eggs of the parasites fall with the feces to the bottom of the sea. There they become embryonated; the zygote develops to form an oncosphere, a hexacanth larva. The eggs of tetraphyllidean species are anoperculate, and the larvae emerge only in the intestine of an appropriate host, typically a copepod. The eggs of tetrarhynchidean species may be operculate; the oncosphere is enclosed in a ciliated covering, and the larva is known as a coracidium. The opercula open and the coracidia emerge. In both groups of tapeworms, the eggs or swimming coracidia are eaten by copepods. The oncospheres migrate from the intestine to the body-cavity of the copepod. There they undergo metamorphosis and transform into procercoids, mobile larvae with tail-like cercomeres in which the larval hooklets are extruded. As noted by Freeman (1973), the change from oncosphere to procercoid is a true metamorphosis, but later developmental stages do not involve a metamorphosis. In both tetraphyllidean and tetrarhynchidean cestodes, when the copepod is eaten and the procercoid is introduced into a second intermediate host, it drops the cercomere and again migrates from the intestine. The second intermediate host may be another invertebrate or a small fish. In it the procercoid develops into a plerocercoid; it may remain in the body cavity but typically the plerocercoid bores into the tissues and becomes encapsulated. If the second intermediate host is eaten by an unsuitable host, the plerocercoid again bores into the body-cavity and a plerocercoid may pass from one intermediate host to another and be re-encysted. Joyeux and Baer (1961, p. 451) distinguished between "hôtes obligatoire" and "hôtes d'attente" or paratenic. The first intermediate host is probably an obligatory species; the second and later hosts may be paratenic. The worms become sexually mature only in the intestine of elasmobranch fishes, sharks and rays.

Pintner (1913) proposed descriptive terms for regions of the metacestodes, and Dollfus (1942) adopted the terminology which has become widely accepted. In tetraphyllidean plerocercoids, the anterior region, *pars antica,* develops into the mature cestode, while the *pars postica* is a bladder-like region that is discarded later. The *pars antica* comprises the *pars bothridialis*, which bears the bothridia, an apical sucker, and a central stalk or peduncle; this portion becomes the scolex and is followed by a *pars proliferus*, the *Anlage* of the strobila. The bothridial region may be invaginated (Fig. 9) by retraction of the retractor muscle which originates in the wall of the *pars postica*. The invaginable region is known as the *pars vaginalis*. In the tetrarhynchid plerocercoid, there is a *pars bothridialis* but no apical sucker; and the stalk is long and contains the tentacles and the bulbs that operate them. The region containing the bulbs is known as the *pars bulbalis,* and its location has taxonomic significance. The *pars postica* is termed the blastocyst, and the *pars antica* may be retracted into it (Fig. 15). A tetrarhynchid that has lost its blastocyst is not comparable to a tetraphyllid plerocercoid and was termed a plerocercus by Dollfus (1942).

Early developmental stages, from the formation of the egg to the formation of the oncosphere, were traced in three tetraphyllidean species: *Acanthobothrium coronatum* Rudolphi, 1819; *A. filicolle* Zschokke, 1888; and *A. zschokkei* Baer, 1948 by Euzet and Mokhtar-Maamouri (1975) and of *Phyllobothrium gracile* by the same authors (1976). The most significant studies on the life-cycle of tetraphyllid cestodes were conducted by Euzet (1959). Eggs of *Phyllobothrium lactuca* van Beneden, 1850, from *Mustelus canis* were isolated in small bowls of aerated sea water. In seven days, at a temperature of 13 to 14 degrees, they contained oncospheres, but movement was feeble. Ten days after isolation, 98% of the eggs contained active larvae and they were placed in a bowl with freshly caught copepods. Eggs and free oncospheres were observed in the intestines of the copepods, and after five days oncospheres were present in the body-cavities of *Acartia clausi* and *Acartia discaudata*. Specimens of *A. discaudata*, examined 16 and 18 days after exposure, contained hexacanth larvae and procercoids of various sizes, some of which measured 200 to 400 μ long. Euzet declared, p. 227, "Cette expérience établit pour la première fois l'existence, pour les Tétraphyllidés marins, d'une larve procercoide chez les Copépodes." He concluded, p. 247, "D'aprés mes observations, les Cestodes peuvent se diviser en deux grands groupes. D'une part, ceux qui possédent une coracidie nageante (*Pseudophyllidea* et *Trypanorhyncha*), et d'autre part, ceux qui n'en ont pas et dont l'embryon hexacanthe sort de l'oeuf dans l'hôte secondaire ou définitif."

The post-embryonic development of various tetrarhynchidean species was studied by Pintner (1893, 1903), Ruszkowski (1932, 1934) and Dollfus (1942). Ruszkowski reported on the life-cycle of *Grillotia erinaceus* (van Beneden, 1858) Guiart, 1927, a parasite of *Raja oxyrhynchus*. The eggs were operculate and after an incubation period of 9 to 10 days, swimming coracidia emerged. They were eaten by copepods and the oncospheres were found in the hemocoel of the crustaceans. The oncospheres transformed and developed into procercoids with cercomeres which contained the larval hooklets. Ruszkowski described later stages from fishes to which the copepods were fed, but apparently did not complete the life-cycle experimentally. Dollfus (1942) reviewed the account of Ruszkowski and described the formation and biology of the plerocercus of *Lacistorhynchus tenue,* using specimens found encysted in the pyloric caeca of *Scomber scomber.* His figure 241 depicts successive stages (a to g) and his Figure g is strikingly similar to Figure 15 of the present study. Dollfus (1942, p. 61) reported, "L'on peut admettre, par analogie avec ce qui a lieu pour les Pseudophyllides d'eau douce, que, lorsque le Copépode (ou peut être un autre invertébré planctonique) porteur du procercoide est ingéré par le deuxième hôte, le procercoide traverse, en abandonnant son cercomère, la paroi du tube digestif de ce deuxième hôte et passe dans la cavité générale. La partie antérieure du procercoide, celle que j'appelle le protéromère, devient plerocercus. Cela n'a pas encore été observé et l'on n'a pas non plus suivi un même individu dans toute la suite du dévelopment postembryonnaire, mais l'on a pu obtenir, pour quelques espèces, la série continue des états successifs par lesquels passe la larve (dans son deuxième hôte) pour arriver à l'état de plerocercus complètement formé avec scolex retiré dans le blastocyste. A l'état le plus jeune observé, le plerocercus en voie de formation apparait comme un massif plus ou moins ovalaire de parenchyme, limité par une cuticle; à un pôle, le parenchyme est plus condensé, avec pullulement de noyaux, c'est le pôle antérieur; au pôle opposé, il y a un pore excréteur auquel aboutit une paire de gros canaux excréteurs. Dans le parenchyme, il y a des ampoules excrétrices à flamme vibratile et des glandes unicellulaires. Il est vraisemblable que, dans ce premier état, le plerocercus est à peine avancé que peut l'être le protéromere du procercoide."

In an abstract, Riser (1951) reported experiments on the life-cycle of *Lacistorhynchus tenue,* and later (1956) he gave a more detailed account. Proglottids from the spiral valve of *Triakis semifasciata* yielded eggs, and coracidia emerged after four to five days of incubation. Oncospheres removed from coracidia were active, moving with hooks opposite the advancing end. Attempts to infect planktonic copepods were successful, but the copepods lived only two or three days. So recourse was taken to splash-pool copepods, *Tigriopus fulvus,* which were easy to rear under laboratory conditions. These copepods became infected, but the exoskeletons were so hard that the procercoids were not liberated in the digestive tracts of the fishes to which they were fed. The copepods were recovered from the feces of the fishes with the procercoids intact. Riser stated that all his material was sent to Dr. R. P. Dollfus for further study.

An interesting experiment was reported by Young (1954). Plerocercoids, identified as *Lacistorhynchus tenue,* were common in the surf-perch, *Cymatogaster aggregata* in San Diego Bay, California. A large, gravid female leopard shark, *Triakis semifasciata,* was autopsied and five unborn young were provided by Dr. Carl L. Hubbs of the Scripps Institution, La Jolla, California. Three young sharks were fed tetrarhynchid larvae from 30 May to 9 July and two were held as controls. One experimental fish was killed on June 4 and contained an immature tetrarhynch. The others were sacrificed July 16. The controls were negative; one experimental fish "contained a few, the other many tetrarhynchs." Whether or not the worms were mature was not stated.

MATERIALS AND METHODS

The squids, *Illex illecebrosus* (Lesueur, 1821) Steenstrup, 1880 (syn. *Ommastrephes illecebrosa*) and *Loligo pealcii* Lesueur, 1821, in New England coastal waters, harbor plerocercoids of tetraphyllidean and tetrarhynchidean cestodes. Squires (1957) reported that specimens of *Illex illecebrosus* from the Newfoundland fishing area were parasitized by larval tapeworms that were identified as *Phyllobothrium* sp. and *Dinobothrium* sp. Brown and Threlfall (1968a, b) reported metacestodes of additional species: *Scolex polymorphus*, *Pelichnobothrium speciosum* and *Nybelinia* sp. from *Illex illecebrosus* in the Newfoundland area. *Loligo pealeii* is rare in the maritime provinces of Canada, although it is the common squid of Cape Cod, Massachusetts. Examination of *L. pealeii* during the summer months of 1974, 1975, 1976 at the Marine Biological Laboratory has disclosed heavy infection with cestode plerocercoids and eight species have been recognized. The squids were taken in the Woods Hole area by the Supply Department of the MBL. During the summer of 1974, a group from the Duke University Medical Center under the direction of Professor T. Narahashi was engaged in neuropharmacological studies using the giant axons of the squids. After the elements essential for the neural investigation were removed, the bodies of the squids were turned over to me for parasitological examination. I wish to acknowledge the kindness of members of the group with special thanks to Dr. Brij Srivastav and Mr. John Starkus.

Between May 25 and August 20, 1974, 128 squids were examined. The digestive tracts contained fragments of food material, bits of fishes, crustaceans, and other remains. In early summer, the stomach and caecum were often stuffed with balls of compacted algae, identified by Dr. Joseph Ramus of Yale University as tips of *Codium fragile*, a species with universal distribution that has appeared in the New England area in the past ten years. Dissection of the squids yielded four species of tetraphyllidean metacestodes and four species of tetrarhynchidean metacestodes, either free in the lumen, attached to the surface, or embedded in the wall of the stomach and caecum. In addition, metacercariae of two species of digenetic trematodes were found; one is a hemiurid which was encysted in the wall of the stomach and the other is a member of the family Didymozoidae with specimens embedded in the walls of both the stomach and caecum.

The worms were studied alive and killed either by the shaking method of Looss or by pressure under a coverglass, depending on the size and texture of the specimen. Fixation was made by the solution of Duboscq-Brasil and the plerocercoids were stained with Ehrlich's haematoxylin, Mayer's paracarmine or Semichon's acetic acid carmine. The Semichon method is somewhat capricious, but when successful, the results are striking, especially in the staining of the hooks of the tetrarhynchs. The metacestodes are described and tentatively assigned to taxonomic groups. Specific descriptions of cestodes are based primarily on sexually mature, strobilate specimens, and since metacestodes are comparable solely with the scolices of adults, determination can be positive only in special cases where features like hook-patterns of tetrarhynchid species are characteristic. The number of plerocercoids present in a squid varied from one to more than one hundred. The small, slightly developed specimens with an apical and four simple, circular suckers (Figs. 9, 10), recognized as *Scolex pleuronectis* O. F. Mueller, 1788, probably identical with *Scolex polymorphus* Rudolphi, 1819, were very common and more than 100 were taken from the stomach and caecum of a single squid. They were free in the lumen or loosely attached to the wall. The larger specimens were present in small numbers, usually less than five. As rule, they were firmly attached to the gut wall or encapsulated in host tissue. When embedded, they could be released only after the cyst wall was cut away.

At my request, representative examples of the several kinds of plerocercoids have been studied by Professor Louis Euzet, Université de Montpellier, France. For his kindness and generosity in examining the specimens and suggesting possible systematic allocations, I express deep appreciation and esteem.

From study of the plerocercoids it is clear that the material contains four different kinds of phyllobothrids and four species of tetrarhynchs. Since definite identification cannot be made, the species are assigned arbitrary numbers and described. Possible taxonomic considerations are discussed.

The specimens described in this report have been deposited in the platyhelminthic collection of the American Museum of Natural History.

Descriptions

TETRAPHYLLIDEA

The tetraphyllidean cestodes have three-host life cycles. The first hosts are copepods in which the oncospheres undergo metamorphosis to form procercoids. Procercoids, in their copepod hosts, are ingested by a second intermediate host, another invertebrate or small fish in which they develop into plerocercoids. Plerocercoids are solid bodied post-larval stages, in which the anterior end may be invaginated and the posterior end may have the beginnings of strobilation. Four species have been taken from *Loligo pealeii* at Woods Hole.

SPECIES NUMBER 1. *Phyllobothrium loliginis* (Leidy, 1887) Linton, 1897. Figures 1, 2.

In the present study, *P. loliginis* was found in 12 squids, free or attached to the wall of the stomach or caecum. Two representative specimens are shown in Figures 1 and 2.

The specimen in Figure 1 is 32 mm long and 3.65 mm in greatest width. The *pars antica* is 17 mm long; the *pars bothridialis* is 2 mm long and the *pars proliferus* is 15 mm long. The scolex is 4 mm wide, the stem is 1.9 mm wide and the apical sucker is 0.18 by 0.20 mm in diameter. The bothridia are sessile, with crumpled and folded edges, 1.5 to 1.8 mm long and 0.09 to 1.00 mm wide. The bothridial suckers are circular to oval and 0.040 to 0.046 mm in diameter. The *pars postica* is 15 mm long and tapers to a point posteriorly. The major excretory tubules are conspicuous. The pore is terminal with a short common duct which divides to form the primary collecting ducts that extend forward ventrally on each side of the body. They enter the scolex, extend around the periphery of the bothridia of that side and pass posteriad, dorsal in location, beside or crossing the ascending tubules. Fluid passes anteriad in the dorsal tubules and posteriad in the ventral ones.

Figure 2 is of the somewhat flattened scolex of a specimen 14 mm long and 2.68 mm in greatest width. In it the *pars antica* is 6 mm long, the *pars bothridialis* is 1.6 mm and the *pars proliferus* is 4.40 mm long. The *pars postica* is 8 mm long. The scolex is 3.10 mm wide; the stem is 1.6 mm wide. The bothridia are separate, slightly stalked, 1.2 to 1.5 mm long and 0.08 to 1.00 mm wide. The apical sucker is 0.19 by 0.20 mm in diameter and the bothridial suckers are oval, 0.04 to 0.046 mm in diameter.

Leidy (1887) described plerocercoids from the northern squid, identified as *Ommastrephes illecebrosa* [= *Illex illecebrosus* (Lesueur, 1821) Steenstrup, 1880], taken at Mt. Desert, Maine as *Taenia loliginis*, and he (1891) predicated that the larva was the scolex of a species in the genus *Tetrabothrium* Rudolphi, 1819 or *Phyllobothrium* van Beneden, 1849. Linton (1897a) reported the worms from the stomach of *O. illecebrosa* taken at Provincetown, Massachusetts, and assigned the species to *Phyllobothrium*. Linton (1901) reported three specimens from the stomach of the silver hake, *Merluccius bilinearis*, collected 11 July, 1900. His figure 231 is indeterminate, but figure 232 is a clear representation of the scolex of *P. loliginis*. Linton (1922b) recorded collection of *P. loliginis* from *O. illecebrosa* and *Loligo pealeii* at Woods Hole, Massachusetts, and the recovery of the plerocercoids from the stomachs of hundreds of fishes examined in the previous twenty years at the laboratory of the U.S. Bureau of Fisheries in Woods Hole. Many of the examinations were made by Vinal N. Edwards. The stomachs typically contained squid, fish, and crustaceans, and it appeared that the plerocercoids were in squid when ingested. Summarizing the report Linton stated, p. 14, "Selachians in which the larval form, *Phyllobothrium loliginis*, was found were *Mustelus canis*, *Squalus acanthias*, *Raja laevis*, and *Raja ocellata*. In no case had proglottides begun to develop. If either of these four species of selachians ever serves as the final host of this cestode the fact is not indicated by any of the data at hand. On the contrary, sufficient numbers of these hosts were examined in the months of July, August, September, October and November without finding any adult cestode that could be linked up with this form to make it appear very improbable that *P. loliginis* can reach adult stage in any of these four selachians.

"There is no reason whatever for thinking that *P. loliginis* ever attains maturity in any teliost (*sic*). It seems, however, that this larval cestode can resist the digestive juices of a great variety of hosts for some time. Hence it doubtless often happens that, before this larval *Phyllobothrium* has reached a true final host, such, for example, as the mackerel shark, or maneater shark, it has sojourned for a shorter or longer time in the alimentary canal of one or more species of selachian, teliost (*sic*) or squid."

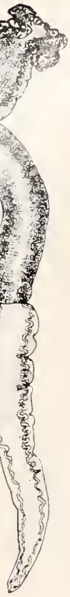
Linton (1922b) described *Phyllobothrium tumidum* n. sp., from the maneater and mackerel sharks, *Carcharodon carcharias* and *Isurus dekayi*, and postulated that it may be the mature stage of *P. loliginis*. The presumption was based on similarity in form and details of structure of the scolex. He noted, p. 15, "In the structure of the bothria and auxiliary suckers, and in the axial portion of the scolex, there is close agreement." One feature which he found disconcerting was the narrow neck region posterior to the bothridia in *P. loliginis*. But this difficulty poses no problem, since the so-called neck region may be contracted and broad as shown in Figure 1 of the present study. Linton noted that the cestodes from *C. carcharias* were mature with detached gravid proglottids in the intestine, whereas "There were no ripe segments" in *I. dekayi*. This observation suggests that *P. tumidum* is a normal parasite of the maneater shark, but that the cestode does not mature in *I. dekayi*. There are strong indications that *P. tumidum* may be the mature stage of *P. loliginis*, and if so the specific name, *tumidum*, will be reduced to a synonym.

The use by Linton of the term bothria for the holdfast organs of *P. loliginis* is unfortunate. Bothria are middorsal and midventral longitudinal grooves on the scolex of pseudophyllidean cestodes. The corresponding structures of the tetraphyllidean cestodes are bothridia, four leaflike outgrowths of the surface, continuous with the underlying tissues. These organs are very mobile and serve for locomotion as well as for adhesion.

Guiart (1933) described and figured a plerocercoid from the mantle of *Ommastrephes sagittatus* taken by Dr. Harant in the Mediterranean. He predicated, p. 465, "A n'en pas douter, il s'agissait de la larve pléroceroide du *Phyllobothrium loliginis* décrit par Leidy en 1887 et en 1890, chez *Ommastrephes illecebrosa* des côtes des États-Unis et revu chez le même hôte par Linton en 1897." Guiart noted that Leidy (1887) had described the plerocercoid as 12.7 mm long and 1.00 mm wide, whereas in the (1891) account Leidy reported larger specimens, as much as 25.5 mm long and 2 to 3 mm wide. Guiart reviewed the description by Linton (1897a) and observed, p. 467, "Une larve si non identique, du moins três voisine, a été décrite en 1929, par R. Dollfus; elle avait été trouvée à Arcachon, par Cuénot, dans la paroi de l'estomac de *Loligo loligo*. Elle mesurait environ 1 cm de long et présentait une tête encore plus caractéristique: en effet, les bothridies sont fortement plissées avec une assez grosse ventouse accessoire en avant et une cinquième ventouse à l'apex du scolex (Fig. 5)." Guiart continued, p. 467, "Justement, j'avais recu autrefois de M. Hérubel un flacon portant l'inscription suivante: 'parasites de *Loligo vulgaris* recueillis par M. Hérubel, à Arcachon, en Mai 1903'. Les parasites étaient encore des larves plérocercoides à corps longuement lancéolé, mais il suffisait d'un examen grossier pour se rendre compte qu'on se trouvait en présence de deux formes différentes, dont l'une, représentée par 8 exemplaires measurant de 10 à 45 mm de longueur, est bien la larve de *Phyllobothrium* sp. trouvée par Cuénot et publiée par Dollfus. La plupart des examplaires mesurent 23 à 30 mm de longueur; la tête mesure 2 mm de longueur sur 2 mm 5 de largeur; l'extrémité du corps, lancéolée, mesure 2 mm 5 dans la plus grande largeur. Les bothridies ont un diamètre moyen de 1 mm. La ventouse accessoire cupuliforme occupe la partie antérieure des bothridies. Enfin l'extrémité antérieure du scolex présente une ventouse."

Commenting on the proposal by Linton (1922b) that *Phyllobothrium tumidum* may be the adult stage of *P. loliginis*, Guiart stated, p. 468, "Il est possible que le *Phyllobothrium tumidum* Linton, 1922 soit la forme adult du *Phyllobothrium loliginis*, mais le fait n'est nullement démontré. En effet, l'aspect du scolex de *P. tumidum* est totalement différent de celui du scolex de *P. loliginis:* la partie antérieure bombée n'existe pas chez la larve et les bothridies rappellent plutôt celles de *Ph. lactuca* que celles de la larve du Calmar. On ne saurait donc actuellement avoir une opinion ferme sur cette question."

Phyllobothrium tumidum Linton, 1922 was redescribed by Riser (1955) on specimens from *Lamna ditropis* Hubbs and Follett taken at Monterey, California and from *Carcharodon carcharias* (Linn.) taken at La Jolla, California. He observed, p. 273, "The species differs from all other members of the genus in that the phyllidea arise posteriorly from the lateral walls of the scolex leaving an anterior cone-like projection visible, the vitellaria extend almost to midline, and the ventral wing of the ovary is absent."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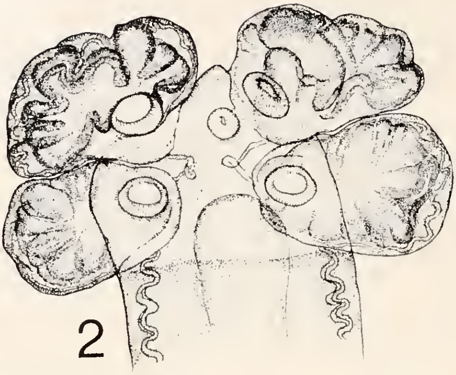
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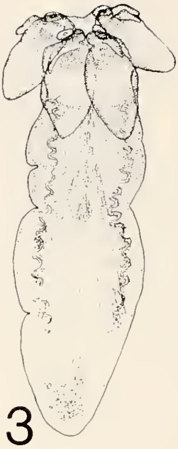
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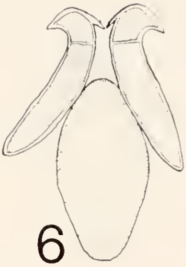
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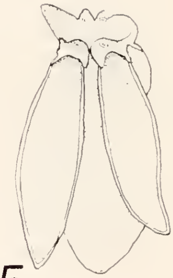
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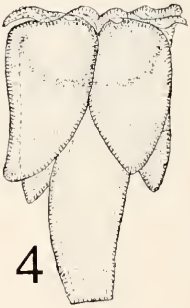
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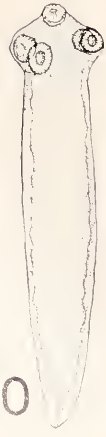
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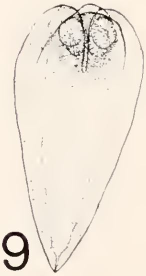
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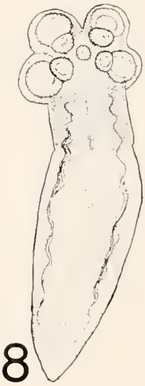
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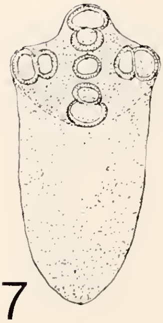
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Squires (1957) reported phyllobothrid and dinobothrid plerocercoids from *Illex illecebrosus* taken in the area between the Grand Banks and the Canadian coast of Newfoundland. Stomach contents of about 1500 specimens were examined in the periods March to November in the years 1946 to 1952. The plerocercoids of *Phyllobothrium* sp. were free in the stomach, caecum, and rectum and often emerged through the anus; those of *Dinobothrium* (*sensu lato*) were often encapsulated in the walls of the caecum and rectum, mostly in the spiral part of the caecum. The phyllobothrids were mainly large, about 20 mm long; the dinobothrids were small, not more than 2 mm long. Infestation by *Phyllobothrium* sp. was greater in small squids taken on the Banks early in the season and gradually diminished as the squids entered inshore waters. The small squid fed mostly on euphasid crustaceans; larger squid on fishes. Incidence of infection by phyllobothrids varied from 35 to 20% from May to November, by dinobothrids it increased from 15 to 75% during this time.

Dollfus (1958) gave a list of the tetraphyllidean larvae that have been reported from cephalopods of the Mediterranean and European Atlantic coasts. He stated, p. 65, "Dans l'estomac, à Arcachon (Gironde), L. Cuénot (in R. Ph. Dollfus 1929) a trouvé un plérocercoide de *Phyllobothrium*. Des spécimens identiques, trouvés aussi chez *L. loligo* (L.), sont conservés au Musée de Münich (voir Ch. Joyeux et R. Ph. Dollfus 1931; R. Ph. Dollfus 1931) et René Legendre en a trouvé plusieurs individus dans l'intestin et la paroi du manteau à Concarneau (Finistère). La même larve que celle de Cuénot a été récoltée en 8 examplaires chez le même hôte, à Arachon, par Marcel Herubel (in Jules Guiart 1933). Cette larve est'sinon identique du moins très voisine', selon Guiart (1933) de *Phyllobothrium loliginis* (J. Leidy 1887), décrit aux États-Unis par J. Leidy (1887, 1891) et Edwin Linton (1897); elle correspond presque sûrement, d'après Linton (1922) "à *Phyllobothrium tumidum* Edwin Linton 1922, identifié adulte chez *Isurus* à Concarneau (Finistère) et à Sète (Hérault)." Williams (1968) reviewed the taxonomy, ecology and host-specificity of some Phyllobothriidae, with a critical revision of *Phyllobothrium*.

The most comprehensive and detailed studies of the Tetraphyllidea are contained in the thèses of Euzet (1959). As noted earlier, he fed eggs of *Phyllobothrium lactuca* from *Mustelus canis* to copepods and removed active procercoids. The summary of the thesis includes the statement, p. 249, "J'ai essayé d'élucider le cycle évolutif encore inconnu de ces Cestodes. D'après mes expériences, les

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FIGURES 1–10. Larval stages of marine metacestodes. The specimens depicted in these figures are deposited in the platyhelminth collection of the American Museum of Natural History under the assigned numbers. Figure 1: *Phyltobothrium loliginis* (Leidy, 1887), *provis.*, specimen, 32 mm long (No. 890). Figure 2: *P. loliginis*, flattened scolex, 3.4 mm wide, specimen 14 mm long (No. 891). Figure 3: *Dinobothrium septaria* van Beneden, 1889 *provis.*, specimen 2.75 mm long (No. 892). Figure 4: *D. septaria*, specimen 0.28 mm long (No. 893). Figures 5 and 6: copies of Figures 10 and 11 in Linton (1897), described as *Thysanocephalum* sp., a larval cestode, 1 mm long, from *Illex illecebrosus* taken at Provincetown, Massachusetts. Dollfus (1936) identified the specimen as *Dinobothrium plicitum* Linton, 1922; probably a synonym of *D. septaria*. Figure 7: *Ceratobothrium xanthocephalum* Monticelli, 1892, *provis.*, specimen 0.56 mm long (No. 894). Figure 8: *C. xanthocephalum*, specimen 2.75 mm long (No. 895). Figure 9: *Scolex pleuronectis* O. F. Mueller, 1788, *provis.*, specimen with invaginated scolex, 0.5 mm long (No. 896). Figure 10: *S. pleuronectis*, specimen extended, 1.2 mm long (No. 897).

Tétraphyllides ne dovient pas posséder de coracidie nageante. Tous les oeufs que j'ai élevés sont morts, 15 à 20 jours après le ponte sans donner cette forme larvaire. J'ai réalisé expérimentalement l'infestation des Copépodes planctonique: *Acartia clausi* Giesb. et *Acartia discaudata* Giesb., avec des oeufs de *Phyllobothrium lactuca* P.-J. Van Ben., 6 jours après l'infestation, j'ai constaté dans la cavité générale des Copépodes des procercoides mesurant de 200 à 400 μ de long. Cette expérience montre pour la première fois les procercoides des Tétraphyllides de sélachiens semblent évoluer dans les Copépodes."

Euzet (1959) described *Phyllobothrium tumidum* from *Isurus oxyrhynchus* taken at Sète in the Mediterranean and at Concarneau on the Brittany coast of France. After studying specimens of the present collection, including those represented in Figures 1 and 2, he reported by letter, "Bien que l'on n'ait aucune preuve expérimentale je suis persuadé que ces formes correspondent à des postlarves de *Phyllobothrium tumidum* Linton, 1922 que l'on rencontre chez les Isuridae."

There is much evidence that *Phyllobothrium loliginis* is a parasite of squids on European as well as American coasts. The final host is almost certainly one or more of the mackerel sharks, Isuridae, wide-ranging fishes that occur in all oceans.

SPECIES NUMBER II. *Dinobothrium septaria* P. J. van Beneden, 1889, *provis.* Figures 3–6.

Only five individuals of this species were found. They were embedded in the wall of the stomach and caecum. The largest and most representative specimen is shown in Figure 3. It is somewhat flattened and the upper bothridia are spread apart, whereas the lower pair of bothridia extend posteriad and their median edges are almost contiguous in the midline. This metacestode is 2.85 mm long. The *pars antica* is 1.33 mm long; the *pars postica* is 1.52 mm long and 0.85 mm wide. The *pars bothridialis* is 0.57 mm long and 0.57 mm wide. The *pars proliferus* is 0.76 mm long and 0.76 mm wide. The anterior face of the scolex is flattened, 0.72 mm wide. There is no myzorhynchus, but the anterior ends of the bothridia are surmounted by muscular crests or bolsters with two fingerlike lobes at each lateral end. The bothridia are sessile, oval, concave, attached by the anterior ends with the posterior ends free and thin. The bothridia bear exceedingly fine spines, and spines three to four times as large are present on the neck region of the *pars bothridialis.* Each bothridum bears an acetabulum, situated near the anterior end, oval in shape and 0.072 by 0.03 to 0.04 mm in diameter. A small specimen is shown in Figure 4. In it the scolex is 0.28 mm wide. The smallest specimen recovered, stained and mounted, is 0.23 mm wide, and it is clear that the plerocercoids increase greatly in size when encysted. The squids are truly intermediate and not mere transfer hosts.

The genus *Dinobothrium* P. J. van Beneden, 1889 was erected to contain *Dinobothrium septaria* van Beneden, 1889, from the mackerel shark, *Lamna cornubica* [= *Lamna nasus* (Bonnaterre, 1788)]. The original description was based on immature worms, and the species was redescribed by Lönnberg (1892, 1899) and by Scott (1909) on specimens from the same host, the porbeagle shark, *L. cornubica.* Certain species assigned to *Dinobothrium* have been transferred to other genera and other species have been suppressed as synonyms of *D. septaria.*

Linton (1922a) described two new species of *Dinobothrium.* Immature specimens taken September 1, 1903 from the spiral valve of a small maneater, *Carcharodon carcharias,* undescribed, but listed as "*Dinobothrium septaria* Linton (*in ms*)" by Sumner, Osburn and Cole (1913), were described as *Dinobothrium plicitum* n. sp. There were ten scolices but the strobilas were immature. In the figure, the bothridia were disposed in cuplike fashion. The scolex was 2.00 mm to 2.50 mm in breadth. Other specimens, taken June 20, 1920 from the spiral valve of a basking shark, *Cetorhinus maximus,* were described as a new species, *Dinobothrium planum.* The scolex of the largest individual was 10 mm broad, 5 mm thick and 8 mm long. In three other specimens the scolices were 6 mm broad and 4 mm thick. Southwell (1925) compared descriptions of *D. septaria* and *D. plicitum,* and referring to *D. plicitum,* declared, p. 170, "This species is inseparable from *D. septaria* van Beneden." Woodland (1927) and Perrenoud (1931) redescribed *D. septaria* and both suppressed *D. plicitum* as a synonym of *D. septaria.* Sproston (1948) included *D. plicitum* as a synonym of *D. septaria* and Baylis (1950) stated, p. 96, "The writer agrees, in the main, with the synonymy given for this species by Sproston." But in describing *D. plicitum* Baylis noted, p. 98, "The status of this species is very uncertain." Linton's (1922a) original material from *Carcharodon carcharias* was immature, and his description contains nothing of diagnostic value concerning the anatomy of the strobila. Guevara (1945) described the species of *Dinobothrium* taken on the Spanish coast. Yamaguti (1952) found *D. planum* in *Cetorhinus maximus* in Japan, and erected a new genus, *Gastrolecithus* to receive the species which was designated, *Gastrolecithus planus* (Linton, 1922).

Euzet (1955) reviewed the history of the genus *Dinobothrium.* He examined the specimens of Sproston, of Baylis, and those from other collections, and gave a revised account of the genus. For *Dinobothrium paciferum* Sproston, 1948, he erected a new genus, *Reesium. Gastrolecithus* Yamaguti, 1952 was named type of a new family, Gastrolecithidae. Concerning *D. plicitum,* he predicated, p. 176, "Une espèce reste douteuse, c'est *Dinobothrium plicitum* Linton, 1922. Le ver était immature et, la différence entre les diverses espèces étant surtout basée sur l'anatomie, il ne peut être mis en synonymie avec l'une ou l'autre. Seule, la récolte chez *Carcharodon carcharias* d'individus adultes pourait trancher la question." But *D. plicitum* may be a synonym of *D. septaria,* and it is possible that *C. carcharias* is not a natural host and that the parasite does not mature in it.

Linton (1897a) reported on a larval cestode. His account, p. 792 reads, "*Thysanocephalum* sp. (Larva). (Plate II, figs., 10–11). One small specimen, 1 mm in length, from the stomach of the squid, (*Ommastrephes illecebrosus*). Collected, August 28, 1886, at Woods Hole, Massachusetts. The squids were caught at Provincetown, Massachusetts (No. 4815, U.S.N.M.) The specmen is the young of my genus *Thysanocephalum,* and presumably of the species *T. crispum.* Only the scolex was present." There was no description of the specimen, but it was depicted in Plate II, figs. 10 and 11. The two drawings are reproduced (Figs. 5 and 6 in the present account) and might have been made from a small individual taken in the current investigation. The plerocercoid clearly belongs to Species Number II. It is very similar and possibly identical with the metacestodes taken by Legendre from squids at Concarneau, France and described by Dollfus (1936) as

Dinobothrium plicitum Linton, 1922. Dollfus (1936) gave a detailed description, with figures, of these plerocercoids and concluded, p. 525, "Signalé deux fois seulement dans la paroi intestinale de *Todaropsis eblanae* (R. Ball) à Concarneau (Finistère) et une fois dans l'estomac d'un *Ommatostrephes illecebrosus* Verrill, à Princetown (sic) Massachusetts. Nous avons préféré conserver le nom spécific de *plicitum* car nous ne sommes pas convaincus de l'identité de cette espèce avec *D. septaria* (voir p. 94)". Baylis (1950) noted that Dollfus (1936) assigned the plerocercoids from *T. eblanae* to *Dinobothrium plicitum* and Sproston (1948) predicated, p. 74, "The recent findings of Dollfus (1936, pp. 523-5) leaves no doubt that the intermediate hosts of *D. septaria* are cephalopods of the family Ommastrephidae." As noted earlier in this report, Squires (1957) found plerocercoids of *Dinobothrium* sp. in squid, *Illex illecebrosus*, in the Newfoundland fishing area. There was no description but the measurement, "not more than 2 mm long", and the figures denote identity with the plerocercoids described by Linton (1897a, 1922a). There is strong probability that the dinobothrid metacestodes from cephalopods on the American and European coasts are conspecific. The observation by Linton (1922a) that they mature only in large, oceanic selachians, which frequent both areas, supports such a postulate.

In the sixth edition of his *Enumération des Cestodes du plancton et des Invertébrés marins*, Dollfus (1964) reviewed the information on plerocercoids from cephalopods. He gave a more detailed account of the morphology of the metacestodes assigned to *D. plicitum* and republished the figures from his (1936) report. His discussion, p. 359, states, "Si l'on s'en tient seulement aux caractères du scolex (et l'on y est obligé lorsqu'il s'agit du plérocercoide), on distingue parmi les spécimens de *Dinobothrium*, d'après les descriptions et figures publiées, deux groupes, différant par un caractère externe très apparent. Chez les spécimens du premier groupe, les deux angles antérieurs du rebord de chaque bothridie se terminent par une pointe courte, rigide, "hook-like" (pour employer l'expression de Linton), à peu près perpendiculaire au plan frontal. Chez les spécimens du second groupe, ces "pseudo-hooklets" n'existent pas.

Le premier groupe d'exemplaires appartient à *Dinobothrium plicitum* Edw. Linton [1922, p. 2–5, 10, pl. I, fig. 1, pl. II, fig. 4–6, de *Carcharodon carcharias* (L.) à Woods Hole, Mass.]. Le second groupe d'exemplaires est representé par *D. planum* Edw. Linton 1922, [p. 2, 5–8, 10, pl. I, fig. 2–3, pl. III-IV, fig. 7-13, de *Cetorhinus maximus* (Günner), à Menemsha Bight, Mass.]."

The relation between *D. septaria* and *D. plicitum* remains uncertain. Clarification of the status of the plerocercoids from *L. pealeii* may be derived from bionomic considerations. Euzet (1959) discussed host-parasite relations. He declared, p. 242, "Je suis persuadé que la spéificité parasitaire des Cestodes est plus large," and on the same page, "Une espèce de Tétraphyllide n'a encore été rencontré que chez une espèce de Sélachiens." In correspondence he has suggested that these plerocercoids are probably referable to *Dinobothrium septaria*, rather than to one of the species from *Cetorhynchus maximus*, since the latter species is a plankton feeder and unable to eat the larger cephalopods. There is a possibility, however, that the cephalopods, like the basking sharks, may have acquired the larvae by ingestion of small crustaceans in the plankton. But *Lamna nasus*, a host of *D. septaria*, occurs at Woods Hole, and the suggestion of Euzet becomes almost a certainty.

SPECIES NUMBER III. *Ceratobothrium xanthocephalum* Monticelli, 1892, *provis.* Figures 7, 8.

Small plerocercoids, recognizable as *Scolex bothriis bilocularis* G. R. Wagener, 1854, were common and scores were taken. Small and larger specimens are shown in Figures 7 and 8. The specimen shown in Figure 7 is 0.56 mm long and the scolex is 0.26 mm wide. The apical sucker is 0.056 mm in diameter and the *pars bothridialis* is very short. Each of the bothridia has a simple border and a bipartate lumen. The overall length of the bothridia is 0.09 to 0.11 mm. The central part of each bothridium is 0.035 to 0.056 mm in diameter and the distal part is 0.05 to 0.07 mm in diameter. Each is somewhat flattened and constricted at the junction of the two parts. Figure 8 shows a larger specimen in which the anterior end is flattened and the bothridia are separated. The *pars antica* is 0.90 mm long; the *pars postica* is 1.85 mm long and 0.85 mm wide. In the *pars bothridialis*, the median peduncle is 0.36 mm long and 0.52 mm wide. The *pars proliferus* is 0.57 mm long and 0.67 mm wide. The apical sucker is 0.11 mm in diameter. The bothridia are 0.36 to 0.042 mm long, with the two sections flattened and slightly constricted at the junctions. The medial portion measures 0.06 to 0.11 mm in diameter and the distal portion 0.20 to 0.28 mm in diameter. There are four small patches of red pigment in the wall posterior to the bothridia.

Linton (1889a, p. 464, Pl. II, figs. 1–12) described *Phyllobothrium thysanocephalum* from the tiger shark, *Galeocerdo tigrinus*. Similar and presumably identical specimens, from an unknown host, in the British Museum were described by Monticelli (1889) as *Phyllobothrium crispatissimum*. Linton (1890, p. 823) erected the genus *Thysanocephalum* with *Thysanocephalum crispum* (= *thysanocephalum*, renamed) as type. Linton (1901, p. 426) listed *T. crispum* from the spiral valve of *G. tigrinus* and p. 430, described *Thysanocephalum ridiculum* n. sp., from the mackerel shark, *Isurus dekayi*. Euzet (1959) corrected the name of the species from the tiger shark to *Thysanocephalum thysanocephalum* (Linton, 1889b), proposed a new subfamily, Thysanocephalinae, in the family Phyllobothriidae, and suppressed *Thysanocephalum rugosum Chandler*, 1942 as a synonym of *T. thysanocephalum*.

Monticelli (1892) described *Ceratobothrium xanthocephalum* n. g., n. sp., from the spiral valve of *Lamna cornubica* (= *L. nasus*) taken in the Mediterranean. Euzet (1959) redescribed *C. xanthocephalum* from specimens taken in *Isurus oxyrhynchus* at the marine laboratories of Sète on the Mediterranean and Concarneau on the Atlantic coast of Brittany, France. He suppressed *T. ridiculum* Linton, 1901 from *Isurus dekayi* as a synonym of *C. xanthocephalum*. Independently, Yamaguti (1959) listed *T. ridiculum* as identical with *C. xanthocephalum* and reported the parasite from *Isurus glaucus* in Japan.

The plerocercoids of Species No. III agree substantially with the descriptions of *C. xanthocephalum*, and while specific determination cannot be based solely on the scolex, they show considerable agreement with the figures of *T. ridiculum* as given by Linton (1901, Pl. XXVII, figs. 294, 295).

SPECIES NUMBER IV. *Scolex pleuronectis* O. F. Mueller, 1788, *provis.* Figures 9, 10.

These plerocercoids were very common and numerous in *L. pealeii* at Woods Hole. Often as many as one hundred were present in a single squid. They conform to the classical representation and cannot be assigned to any genus or species. Fixed, stained, and mounted specimens are shown in Figures 9 and 10. Figure 9 is of a specimen in which the anterior end is invaginated; it is 0.50 mm long and 0.23 mm wide. Figure 10 is of a specimen 1.2 mm long and 0.20 mm wide. The apical sucker is 0.07 mm in diameter and the bothridia are represented as simple, circular suckers, 0.09 to 0.10 mm in diameter.

Under the designation, *Scolex pleuronectis*, there has been recorded a variety of larvae, all apparently tetraphyllidean, taken from a large list of invertebrates and the intestine of fishes that had ingested the former hosts. Linton (1901) reported *Scolex polymorphus* from 28 species of teleost fishes examined at Woods Hole. Reporting on parasites of fishes taken at Beaufort, North Carolina, he observed, (1905, p. 326) "The larval cestodes, doubtless representing several different genera, recorded in Parasites of the Woods Hole Region under the name *Scolex polymorphus*, were found in 34 of the 59 Beaufort fishes examined (Figs. 76–79). As at Woods Hole, these forms are found not only in the alimentary tracts of their hosts, but also in the cystic ducts of several. They are almost never absent from the cystic duct of *Cynoscion regalis*. In all cases where these worms have been obtained from the cystic duct and from the intestine of the same fish, those coming from the cystic duct are larger, plumper, and more opaque than those from the intestine. Some of the older larvae suggested the genera *Calliobothrium*, *Acanthobothrium*, and *Phoreiobothrium*."

Discussing the group of plerocercoids known as *Scolex pleuronectis*, Wardle and McLeod (1952, p. 231) predicated, "It is probable that these larvae represent a number of tetraphyllidean species." Euzet (1959, p. 228) reported, "On recontre *Scolex pleuronectis* dans le tube digestif de nombreux Téléostéens marins aussi chez un certain nombre d'invertébrés: Cténophores (*Scolex acalepharum* M. Sars, 1845; *Tetrastoma playfairi* Forbes, 1849; Vers Nemertes (*Scolex*); Mollsuques Lamellibranches (*Scolex*); Mollusques Céphalopodes (*Scolex pleuronectis* O. F. Müller); Crustacés Copépodes (*Plerocercoides aequoreus* Wündsch et *Plerocercoides armatus* Wündsch, 1912); Crustacés Décapodes (*Scolex paguri bernhardi* K. M. Diesing, *Scolex carcini maenadis* Vaullegeard, *Echeneibothrium mouchetae* R.-P. Dollfus). Cette larve très polymorphe même dans un même hôte, est très difficile à décrire et il est certain que les larves de différents Tétraphyllides ont été groupées sous un même nom. Ces parasites sont en général aplatis, lancéolés vers l'arrière et présent, à la partie antérieure, 4 bothridies et une ventouse apicale."

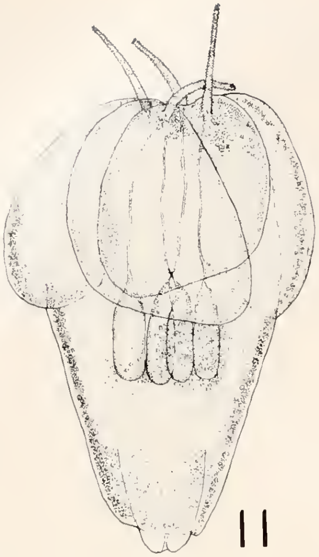
TETRARHYNCHIDEA

Tetrahynchid metacestodes are post-larval forms that have lost their blastocysts and, accordingly, are not directly comparable to the plerocercoids of the Tetraphyllidea. For this stage, Dollfus (1942) proposed the designation, plerocercus.

SPECIES NUMBER V. *Nybelinia bisulcata* (Linton, 1889) Dollfus, 1929, *provis.* Figures 11, 12.

These worms were embedded in the walls of the stomach and caecum of squids. The cysts were tough, fibrous, and the metacestodes were released only when the cyst was dissected away. The specimens are ovate, wider anteriorly. The anterior end is rounded, often somewhat flattened. The specimens are very uniform in size, the total length varies from 3.40 to 3.90 mm and the width from 2.20 to 2.40 mm. The greatest width is at the posterior portion of the bothridia, termed pseudobothridia by certain authors. In length, the *pars bothridialis* measures 1.80 to 1.90 mm and the *pars postbothridialis* is about the same length. The *pars bulbosa* measures 0.60 to 0.80 mm in length and the anterior end is partially overlapped by the *pars bothridialis.* The region between the *pars bulbosa* and appendix is 0.40 to 0.50 mm in length. The *pars post bulbosa* is 0.60 to 0.75 mm in length, and the length of the velum is determined by the degrees of retraction of the *appendix.* The posterior end of the appendix bears the excretory pore; there is a short vesicle and the collecting ducts pass forward on either side of the structure. The tentacular bulbs are banana-shaped, bent slightly outward at the middle. They measure 0.55 to 0.75 mm in length and 0.20 to 0.25 mm in width. The tentacle-sheaths are longer than the bulbs or the tentacles. The tentacles are 0.80 to 0.85 mm long when fully extended. Without hooks, they are 0.08 to 0.09 mm wide at the base and 0.03 to 0.35 mm wide at the tip. They do not have a special armature at the base. The hook arrangement is homeoacanthous (Fig. 12). The hooks are solid; they measure 0.018 to 0.021 mm long and 0.007 to 0.008 mm wide at the base. Those near the base of the tentacles are slightly shorter, abruptly recurved, without extended bases; proceeding toward the tip the hooks are slightly longer, with sharper points and broad elongate bases. The hooks are set in diagonal spiral rows, with six or seven visible in each row. The rows are separated by intervals, each about one-half the length of the hooks, and appear to cross like the letter X, when the plane of focus in shifted from one side to the other. There are about 30 rows of hooks. The tegument of the body bears fine, sharply pointed, flat spines, 10 to 14 μ long and 3 μ wide. In addition, the edges of the bothridia are thickened and bear longitudinal rows of closely set bristles, 25 to 30 μ long. They are uniform in diameter and form a fringe that resembles cilia. The body wall consists of circular, longitudinal and oblique muscle fibers and below the wall the parenchyma is filled with secretory cells. The secretion appears in the form of vesicles, 12 to 20 μ in diameter, partially filled with fluid or scores of minute granules.

Cuvier (1817) described tetrarhynchid larvae from the turbot, (= *Scophthalmus maximus*) *as Tetrarhynchus lingualis.* Similar larvae from the mantle and stomach of *Sepia officinalis* taken at Nice, France were identified by Wagener (1854) as *Tetrarhynchus lingualis* Cuvier, 1817. Vaullegeard (1899) described metacestodes from connective tissue cysts in the wall of the digestive tract of *Octopus vulgaris,* taken off the coast of Brittany as *Tetrarhynchus bisulcatum* Linton, 1889. The specimens were 3 to 4 mm long, the tentacular bulbs measured 0.34 by 0.11 mm, and the hooks were 0.020 mm long. Tentarhynchid larvae were taken, mainly by Professor Cuénot, from the stomach wall of *Loligo loligo, Sepia officinalis,* and *Sepia fillionxi* at Arcachon, France and studied by Dollfus (1929). He described the specimens from *Sepia fillionxi* as *Nybelinia lingualis* (Cuvier). He declared that the plerocercoids from cephalopods do not belong to the genus *Tetrarhynchus,* but to the genus *Nybelinia* Poche, 1926, a new name for the genus *Aspidorhynchus* Molin, 1858, preoccupied. Dollfus (1930) discussed the "groupe *lingualis-bisulcatus*" and stated, p. 208, "Ne m'occupant pas ici d'une révision des espèces, je



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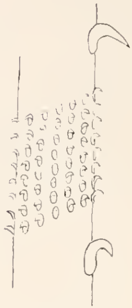

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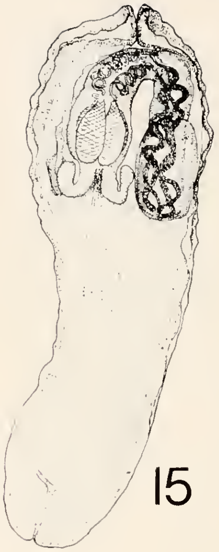

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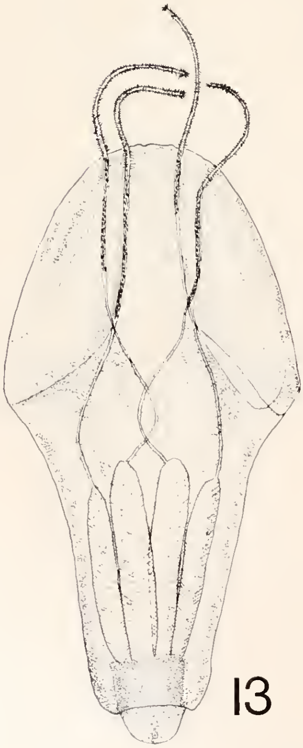

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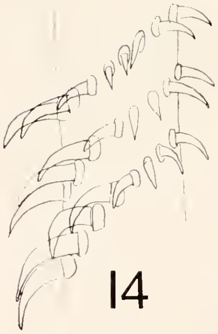

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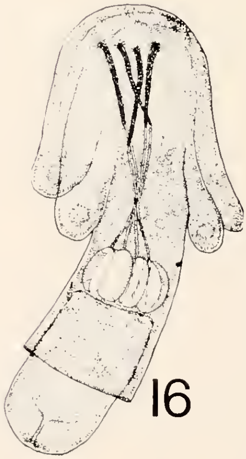

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n'examinerai pas si *bisulcata* (Linton) tombe, ou non, en synonymie avec *Linguale* (Cuvier) ou n'en est qu'une variété." Dollfus (1942) listed the synonymy of *Nybelinia* and divided the genus into two subgenera: *Nybelinia* and *Syngenes. Nybelinia lingualis* (Cuvier) was designated type of the subgenus *Nybelinia* and a key was formulated to the ten species recognized as valid. In the Key, p. 146:
"B. Formes dont les plus grands crochets n'atteignent pas 40 μ.
B2 Longueur des bulbes de plus du tiers à environ la moitié de celle des bothridies.
B$_2$b Plus grands crochets 20–24 μ. Gaines plus courtes que les bothridies.

Pars bulbosa en partie comprise dans *pars bothridialis.*

Velum court, plus court que chacune des autres parties du scolex, commencant assez loin au arrière des bulbes. *N. bisulcata* (E. Linton, 1889)."
The metacestodes from *L. pealeii* conform to the diagnosis of *N. bisulcata.*

Linton (1889a) described and figured tetrarhynchid cestodes from the dusky shark, *Carcharinus obscurus,* as a new species, *Rhyncobothrius bisulcatum.* He (1897a, p. 810) transferred the species to the genus *Tetrarhynchus.* In his report on larval cestode parasites of fishes, Linton (1897a) described and figured encysted larvae, identified as *Tetrarhynchus bisulcatum,* from a variety of hosts, including *Paralichthys dentatus, Cynoscion regalis, Tetranarce occidentalis, Stenotomus chrysops,* and *Seriola zonata.* The cysts were located principally in the mucous and submucous layers of the stomach. He noted, p. 810, "I have found it very abundant in the squeteague at Woods Hole, Massachusetts, and have seldom examined one of these fish without finding numerous examples of encysted tetrarhynchs in the stomach walls." The description and figures of these larvae (Pl. VI, Figs. 11–15) are so similar to the metacestodes from *L. pealeii* that their identity is apparent. Linton observed, p. 787, "The finding of a larval cestode parasite encysted in the tissues of a fish is not always proof that the fish is a true intermediate host. Beneden invented the term *xenosite* i.e., stranger-for this condition." Baer (1951, p. 12) designated such hosts as "hôtes d'attente" or paratenic hosts. Referring to *T. bisulcatus,* Linton (1897b) reported, p. 452, "I have found this species in the adult condition, thus far, only in the dusky shark (*Carcharhinus obscurus*)."

Linton (1924) reviewed his studies on the cestode parasites of sharks and skates. Discussing the species, *Tetrarhynchus bisulcatus* (Linton), he reported adult worms from *Carcharhinus obscurus* and *Carcharhinus milberti* with single specimens from the stomach of *Galeocerdo arcticus* and *Squalus acanthias.* Encysted larval stages were recorded from 18 species of fishes. As noted earlier, Dollfus (1929) transferred the species, *T. sulcatus* to *Nybelinia.* The finding of encysted metacestodes in both cephalopods and teleost fishes shows that these are merely paratenic hosts that acquired the parasites by ingestion of previous hosts and that the worms become mature only in the intestine of selachian fishes. The

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FIGURES 11–16. Larval stages of marine metacestodes. The specimens depicted in these figures are deposited in the platyhelminth collection of the American Museum of Natural History under the assigned numbers. Figure 11: *Nybelinia bisulcata* (Linton, 1889) *provis.*, specimen 3.80 mm long (No. 898). Figure 12: section of a tentacle of specimen No. 898. Figure 13: *Nybelinia yamagutii* Dollfus, 1950, *provis.*, specimen 5.00 mm long (No. 899). Figure 14: section of tentacle of specimen No. 901. Figure 15: *Lacistorhynchus tenue* (van Beneden, 1958) *provis.*, specimen 1.52 long (No. 900). Figure 16: *Otobothrium crenacolle* Linton, 1890, *provis.*, specimen 1.40 mm long (No. 901).

fact that the final hosts are large, oceanic fishes explains the wide dispersal of the parasite.

SPECIES NUMBER VI. *Nybelinia yamagutii* Dollfus, 1960, *provis.* Figures 13, 14.

Five plerocerci of this species were removed from cysts in the wall of the stomach. The worms are similar in size and form, ovate, rounded to slightly flattened anteriorly, tapering posteriorly. They measure 4.3 to 5.0 mm in length and 1.90 to 2.30 mm in width. Greatest width is at the level of the bothridia, which are sessile with attached edges. The *pars bothridialis* is 1.75 to 2.00 mm long, the *pars postbothridialis* is 2.10 to 2.70 mm long. The *pars bulbosa* is about two-thirds as long as the *pars bothridialis.* It is distinctly posterior to the *pars bothridialis* and extends to the appendix. The appendix is 0.20 to 0.50 mm long; the velum is 0.20 to 0.30 mm long. The tentacular bulbs are 1.45 to 1.55 mm long and 0.22 to 0.28 mm wide. They are cylindrical, slightly attenuated at the ends and extend from the appendix about four-fifths of the distance to the *pars bothridialis.* The tentacle-sheaths are longer than the bulbs and shorter than the tentacles, which when fully extended may be 2.30 mm long. The tentacles, without hooks, are about 0.12 mm wide and there are about 50 rows of hooks. The hooks near the base of the tentacle are smaller with a special arrangement, and they increase in size toward the tip. One side of the tentacle bears small hooks, the other side bears large hooks (Fig. 14). The small hooks have oval bases, about 0.020 by 0.012 mm and are 0.030 to 0.035 mm long. The large hooks have longer and broader bases and measure 0.080 to 0.085 mm in length. The hooks stain intensely with the Semichon technique. The hook pattern is almost identical with that in the species described by Dollfus (1960) as *Nybelinia yamagutii.* It was based on postlarvae from the general cavity of *Liosaccus cutaneus* (Günther), a teleost taken off the coast of Dakar, West Africa. The description and figure by Dollfus show the tentacular bulbs extending to the bothridia, but the wall of the *postbothridialis* is crenate, which indicates retraction of the region with forward protrusion of the bulbs. The name *N. yamagutii* was selected because Yamaguti (1934) had described a similar species, *N. pintneri,* from the blue shark, *Prionace glauca,* taken on the Pacific coast of Japan.

In Species No. VI, the tegument contains fine, flattened spines; the edges of the bothridia bear longitudinal rows of long, closely set, cylindrical bristles; and the parenchyma is filled with secretory cells. In these respects it agrees with Species No. V, but they differ in the relative length of the postbothridial region, in the attached edges of the bothridia, in the location and length of the tentacular bulbs, and in the armature of the tentacles.

SPECIES NUMBER VII. *Lacistorhynchus tenue* (van Beneden, 1858) Pintner, 1913, *provis.* Figure 15.

Three specimens were recovered from the washings after dissection of the stomachs and caeca of squids. They are identified as plerocerci of *Lacistorhynchus tenue* (van Beneden, 1858), the only recognized species in the genus. Pintner (1913) erected the genus *Lakistorhynchus* with *Tetrarhynchus tenuis* P. J. van Beneden, 1858 as the type species. He listed the species *T. benedenii* Créty, 1890 and *T. gracile* Diesing, 1863, as synonyms. The species, *T. benedenii* was included originally in *Dibothriorhynchus* Diesing, 1850, and was transferred to *Tetrarhynchus* by Vaullegeard (1899). The species, *tenuis*, was renamed, *gracile*, and included in *Rhynchobothrius* Rudolphi, 1819 by Diesing (1863). Subsequent authors have consistently written the name *Lacistorhynchus tenuis* (van Beneden, 1858) Pintner, 1913. But Yamaguti (1959) determined that the Greek word, *rhynchos*, is a neuter noun and he adopted the spelling, *tenue*, for the name of the species.

The specimens had emerged from their cysts, but the blastocysts are intact and the plerocerci agree in all respects with the figure of this stage given by Dollfus (1942, Fig. 241, g). In all of them the scolex is retracted, bent in an inverted U-shape, and occupies from one-third to two-fifths of the total length. They are slightly smaller than the specimens described by Dollfus, who reported that the plerocerci become smaller as they mature and the definite structures are formed. The present specimens are 1.5 to 1.80 mm in length and 0.50 to 0.55 mm in width. There are two bothridia, notched posteriorly, 0.23 to 0.25 mm long and 0.15 to 0.17 mm wide. The tentacular bulbs are 0.15 to 0.18 mm long and 0.05 to 0.06 mm in diameter. The postbulbar appendix is 0.10 to 0.12 mm in length. The distance from the tentacular bulbs to the bothridia is 0.45 to 0.55 mm. The tentacular sheaths are coiled and since the tentacles are retracted, the armature can not be determined in detail. The hooks are apparently arranged in a chainette and extend almost all the way to the bulbs. The tegument bears fine spines and the blastocyst measures 0.80 to 0.90 mm in length and 0.35 to 0.40 mm in width.

These metacestodes have long been known. According to Dollfus (1942, p. 321), "La larve de ce tétrarhynque—en raison de la forme particulière qu'affecte généralement son kyste, comme aussi en raison de sa grande fréquence (étant rencontrée souvent par dizaines et même par centaines) dans les poissons comestibles les plus communs de nos marchés—a, depuis longtemps, attiré l'attention des helminthologistes." They were described by Deslongchamps (1824), and the strobilate stage of the species was described by van Beneden (1850) from *Galeus canis* taken at Ostende. Van Beneden (1870) predicated the identity of the two stages. The most complete account of the morphology, life-cycles, and taxonomy of these cestodes is contained in the monograph by Dollfus (1942).

Linton (1889a) described *Tetrarhynchus tenuicolle* Rudolphi, 1819 from *Mustelus canis* at Woods Hole, Massachusetts. He (1889b) decided that the species he had identified as *T. tenuicolle* was actually a new species, which he described as *Rhynchobothrium bulbifer*. The next year, (1890) he described a second species, *Rhynchobothrium heterospine*, which differed from *R. bulbifer* in the absence of a bulb behind the scolex. Linton (1924) reported adult stages of *R. bulbifer* from *Galeorhinus laevis, Squalus acanthias,* and *Vulpecula marina*. He noted, p. 58, "The cysts and plerocerci of this species are often club-shaped, or gourd-shaped, the larger end being subglobular, the remainder, which is of much smaller diameter and cylindrical, may be either straight or curved. Usually encysted on viscera, and in intestinal wall, but common in the flesh of sand eels, silverside, and young herring." The cysts were recorded from 35 species of fishes in the period 1895–1924. The larval stage of *R. heterospine* was reported from four species of fish. Vaullegeard (1899) announced the identity of *R. bulbifer* and *R. tenuis* van Beneden, 1858. Pintner (1913) noted the similarity between *R. hetero-*

spine Linton, 1890 and *R. tenuis*, and Dollfus (1942) after examination of many larval and adult specimens declared the identity of the three species, *R. tenuis*, *R. bulbifer*, and *R. heterospine*.

SPECIES NUMBER VIII. *Otobothrium crenacolle* Linton, 1890, *provis.* Figure 16.

Two small metacestodes, found in the washings of dissections of stomach and caeca of squids are referred to this species. The specimens measure 1.2 and 1.4 mm in length; 0.5 and 0.56 mm in greatest width. They are rounded anteriorly and widest at the level of the posterior ends of the bothridia. The bothridia are patelliform, longer than wide, convergent anteriorly and divergent posteriorly, with deep median incisions and free ends. They are 0.60 to 0.67 mm long and have thickened edges that bear longitudinal rows of closely-set, long, cilia-like bristles. Lightly staining circular areas near the posterior ends of the bothridial lobes probably represent the described eversible, ciliated pits. The tegument contains very small flattened spines. The tentacular bulbs are oval, 0.12 to 0.15 mm long and 0.036 to 0.042 mm wide. They are situated at the posterior end of the scolex and do not extend forward to the level of the bothridia. The tentacular sheaths are longer than the tentacles, which are almost completely retracted and in this condition measure 0.26 to 0.29 mm long and 0.012 to 0.015 mm wide. The armature is heteraconth, with alternate rows of large and small hooks but only the spines at the anterior ends of the sheaths are clearly visible and the pattern is indistinct. The large hooks are curved with firm bases, 10 to 12 μ long; the small hooks are 3 to 4 μ long. The velum is 0.15 to 0.22 mm long; the appendix is 0.24 to 0.35 mm long and 0.26 to 0.30 mm wide. The shape and size of the tentacular bulbs distinguish these specimens as *O. crenacolle*.

Dollfus (1942) erected a new family, *Otobothriidae* to contain *Otobothrium* Linton, 1890 and *Poecilancistrium* Dollfus, 1929. He gave a more precise generic diagnosis of *Otobothrium* and proposed a new subgenus, *Pseudotobothrium*, to receive *Otobothrium dipsacum* Linton, 1897. Discussing the genus he predicated, p. 239, "Il y a quelques espèces encore très mal connues, qui ne peuvent pas être classées avc certitude dans un des deux sous-genres. Parmi les dix espèces admises ici dans le genre *Otobothrium*, il y en a vraisemblablement de synonymes; les descriptions publiées sont incomplètes et beaucoup de figures sont insuffisantes, surtout en ce qui concrne l'armature des trompes." These worms are parasites of wide-ranging oceanic fishes, and Linton, (1934) commenting on the distribution of helminth Entozoa of fishes of the Woods Hole region (Massachusetts, U.S.A.), stated, p. 130, "While the adult stages of selachian cestodes are limited to one or a few hosts, the encysted stage appears to be tolerated in any fish in which it may obtained lodgement. Thus, the species *Otobothrium crenacolle* Linton, has been found in cysts, usually on the viscera or in the submucosa of the stomach or intestine, of 16 species of fish, including four species of shark, and *Otobothrium imparispine* Linton has been recorded from 37 species of teleosts."

Summary

Metacestoid stages of tetraphyllidean and tetrarhynchidean cestodes were described from marine fishes by O. F. Mueller (1787), Fabricius (1794), and

Cuvier (1817). Although known for more than a century, knowledge of these tapeworms is meager, and the life-cycle has not been experimentally demonstrated for any one of them. The worms mature only in the spiral valve of elasmobranch fishes, but larval and developmental stages occur in a great variety of marine invertebrates as well as in fishes. Technical difficulties of maintaining experimental hosts for long periods under controlled laboratory conditions have precluded the successful completion of life-histories. After a period of incubation, the eggs contain motile larvae, oncospheres, which, when eaten by copepods, migrate to the body cavity and transform into second stage larvae, procercoids. When the copepods are eaten, the procercoids emerge from the intestine of the second intermediate host and enter the body cavity or penetrate adjacent tissues where they develop into plerocercoids and may be encapsulated. If the second intermediate host is eaten by other than an elasmobranch, the plerocercoids may again emerge from the intestine and be re-encysted. Thus, a plerocercoid may pass through a series of intermediate hosts before arriving at a suitable selachian host. Such residence in successive paratenic hosts provides for long life for the individual and wide distribution and dispersal of the species. The life-cycle, accordingly, is essentially a food chain, with a shark, skate, or ray serving as the final link.

Data compiled from literature encompasses the biology and bionomics of these cestodes, their development, intermediate and definitive hosts, taxonomy and nomenclature. Descriptions are presented of eight species of metacestodes from squids taken in the Cape Cod area. They are provisionally assigned to species, four in the order Tetraphyllidea and four in the order Tetrahynchidea.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

Abstracts are arranged alphabetically by first author. Author and subject references will be found in the regular volume index, appearing in the December issue.

GENERAL SCIENTIFIC MEETINGS
August 22–26, 1977

Effects of KCl concentration on the interaction of F-actin and tropomyosin: a sedimentation velocity study. Fumio Arisaka.

The KCl dependent association of tropomyosin (TM) with F-actin was found to vary drastically with the method of protein sample preparation. Simple mixing (in the test tube) of TM and actin in a solution containing 0.1 M KCl produced a large aggregate (>200 S) which resulted from the nonspecific aggregation of these proteins. To avoid this problem, the final concentration of KCl in TM-actin mixtures had to be adjusted by dialysis. Sedimentation velocity studies of dialyzed TM-actin mixtures with a TM/actin molar ratio of 0.12 indicated that no interaction of TM with actin occurred at concentrations of KCl below 25 mM. The degree of association increased to 100% as the KCl concentration was increased to 0.1 M. By varying the molar ratio of TM/actin from 3.1 to 0.41 in the initial mixture (at 0.1 M KCl), it was found that the molar ratio of TM bound to actin reached a plateau value of 0.3, indicating that the interaction of TM with actin in this system was specific. Using this dialysis procedure, it should now be possible to study the interaction of TM and actin in the ultracentrifuge.

This work was supported by grant GM-00265 from USPHS to the Physiology Course.

Motility of the Limulus *amebocyte.* Peter B. Armstrong.

The cell found in the coelomic fluid of the horseshoe crab, *Limulus polyphemus*, can be stimulated to become motile by extravasation or trauma. Motility was studied using time-lapse microcinematography and direct microscopic examination of cells in tissue culture chambers and in isolated gill leaflets from young animals. In both situations, motile cells show two interconvertable morphological types: the contracted cell, which has a relatively small area of contact with the substratum, and a flattened form with a larger area of contact. In both cell types, motility involves the protrusion of hyaline pseudopods followed by flow of granular endoplasm forward into the pseudopod. Cellular motility *in vivo* (in the gill leaflet) is morphologically identical to that displayed in tissue culture. Granule flow, which is important in producing forward movement of the posterior end of the cell, might be produced by either circumferential or longitudinal contraction of the posterior end of the cell as a whole or might be generated by the interaction of the individual granules with formed elements (microtubules or microfilaments) in the endoplasm. The observation that individual granules in flattened cells sometimes display considerable independence in rate and direction of movement suggests that granule flow is not produced by contraction of the posterior portion of the cell. Since cell motility is unaffected by high concentrations (200 μg/ml) of the microtubule-depolymerizing agent colcemid, microtubules appear not to be involved in granule flow. Motility is abolished by cytochalasin B at 1 μg/ml.

This work was supported in part by Cancer Research Funds of the University of California.

Changes in protein phosphorylation following fertilization of Arbacia punctulata *eggs.* Tom Aune and Tim Hunt.

Enzyme modification by addition or removal of phosphate groups is known to be the basis of several control mechanisms in cells. Such a mechanism may be important in activation of metabolism in sea urchin eggs after fertilization. *Arbacia* eggs were incubated in artificial sea water with 0.5 mCi/ml $^{32}PO_4$ for two hours before washing and fertilization. Approximately 20% of the $^{32}PO_4$ taken up by the eggs was identified as $AT^{32}P$, whose specific activity did not change during the experiment. Eggs were sampled before and after fertilization and analyzed by SDS-polyacrylamide gel electrophoresis. Protein bands were visualized by Coomassie staining and labeled bands by autoradiography.

A major protein of apparent molecular weight of 31,000 daltons was phosphorylated 5–10 min after fertilization. Subcellular fractionation experiments indicated that this protein sedimented with ribosomes at $100,000 \times g$. Minor proteins of 21,000 and 41,000 daltons were also phosphorylated 2 min after fertilization. However, phosphorylation of the 21,000 dalton protein was not complete until 20 min after fertilization. A 98,000 dalton protein appeared to be dephosphorylated 5 min after fertilization and re-phosphorylated prior to first cleavage. Parthenogenic activation by ionophore A23187 resulted in phosphorylation of the 31,000 dalton protein and a new protein of 105,000 daltons.

Intracellular pH increases from 6.7 to 7.3 immediately following fertilization. Two changes in the pattern of phosphorylation could be induced by altering the pH of cell-free egg homogenates. At pH 6.7 the 98,000 dalton protein was phosphorylated while the 105,000 dalton protein was not phosphorylated. At pH 7.3, the 98,000 dalton protein was not phosphorylated but the 105,000 dalton protein was phosphorylated. The physiological significance of these changes, while suggestive, is at present unknown.

This work was supported by NIH training grant HD-07098.

Efferent inputs and serotonin enhance the sensitivity of the Limulus *lateral eye.* ROBERT B. BARLOW, JR., STEVEN C. CHAMBERLAIN AND EHUD KAPLAN.

Efferent activity in the optic nerve trunk mediates circadian changes in the sensitivity of the *Limulus* eye. At night the efferent inputs elevate the electroretinogram (ERG) and optic nerve responses and lower the spontaneous optic nerve activity (SA). These effects reverse during the day when the circadian efferent activity stops. The effects of the efferent inputs, however, can be produced during the day by shocking the cut optic nerve trunk *in situ.* Different shock regimes permit separation of the effects. For example, shocking the nerve trunk for 30 sec at a frequency of 4 shocks/sec abolished SA for several minutes but had no effect on the ERG. SA returned to preshock levels in about one hour. Shocking the nerve repetitively for 2 hr at 4 shocks/sec for 30 sec every minute was required to maximally elevate the ERG amplitude. After shock offset the ERG declined to preshock levels with a time constant of 45 min and the SA fully recovered within 2 hr. The reduction of SA by efferent inputs to the eye appears to originate in the photoreceptors (retinular cells). Shocking the optic nerve reduced the frequency of spontaneously generated quantum bumps by more than 90% without reducing the efficacy of the photoreceptors to elicit light-evoked bumps.

Serotonin mimics the effects of the efferent inputs on both the ERG and SA. Injection of 20 μl of serotonin (0.5 mM) under the cornea of the eye *in situ* lowered SA for about 3 hr and elevated the ERG by 200%. The effects of serotonin were reversible, graded with concentration, and followed time courses similar to those of the effects of nerve shock. The results indicate that the efferent inputs and serotonin enhance the sensitivity of the *Limulus* lateral eye by lowering photoreceptor noise (spontaneous quantum bumps) and elevating photoreceptor response (ERG amplitude).

Supported by the Grass Foundation and by NIH grant EY-00667.

Theory of a method for determination of sperm motility. CHARLES P. BEAN.

Methods for determination of sperm motility include photography of individual cells (Rothschild and Swann, 1949, *J. Exp. Biol.*, **26**: 164–176; Gray, 1955, *J. Exp. Biol.*, **32**: 775–801) or electronic analysis of a moving spot in the image plane of a microscope (Rikmenspoel, 1962, pp. 31–54 in D. W. Bishop, ed., *Spermatozoan Motility*). These procedures are somewhat tedious to apply and require the analysis of many individual pictures or events to deduce an average motility. I describe a method that—to the extent its assumptions are fulfilled—gives a true average motility. The assumptions are: first, the sperm move in straight lines until colliding with a surface; and secondly, at least one surface adsorbs incident sperm in an irreversible fashion. A dilute suspension of sperm (N in a unit volume with average velocity V) is put in a layer of thickness, h, over an adsorbing surface and the surface density noted as a function of time, t. For times less than h/V, the surface density is given exactly as NVt/4. For very long times, all the sperm adsorbs to the surface and the surface density approaches a limiting value, Nh. The course of sperm attachment between these limits depends in a calculable way on the distribution of velocities and scattering conditions at the nonadsorbing air-suspension interface.

This work was stimulated by the summer Embryology Course, David Epel and Tom D. Humphreys, co-directors. The course was supported by NIH Training Grant HD-07098.

The pupillary response of flies as an optical probe for determining spectral sensitivities of retinular cells in completely intact animals. GARY D. BERNARD AND DOEKELE G. STAVENGA.

The spectral sensitivity of the peripheral retinular cells in six species of intact flies was determined using non-invasive, optical measurements of the pupillary response. This new approach to measuring sensitivity of retinular cells in compound eyes builds upon the work of N. Franceschini (1972, pp. 75–82 in R. Wehner, ed., *Information processing in the visual systems of arthropods*), who demonstrated the feasibility of measuring the action spectrum of pupillary scattering, and upon our previous studies of insect visual pigments and pupillary responses. Our technique is to chronically illuminate a localized region of the eye with a long-wavelength beam, adjusted to bring pupillary scattering above threshold, then, after stabilization, to stimulate with monochromatic flashes. A criterion increase in scattering is achieved by adjusting flash intensity. All spectral sensitivity curves exhibit a major peak at 360 nm or less, a minimum of at least 0.4 log-units at 400 nm, and a somewhat lower peak at 450 nm or greater. The curves fall into three groups based on the wavelength of the secondary peak; a) 490 to 500 nm—for a muscid fly and the dorsal region of a syrphid; b) 475 nm—for a chloropid and an ephydrid; c) 450 nm—for the ventral region of two syrphids and a bombyliid. The syrphid eye is globally non-uniform. The dorsal pole contains receptors which peak at 490 nm, whereas those in the ventral pole peak at 450 nm. We investigated the possibility of local non-uniformity by measuring single cell-types (R1 and R6, R2, R3, R5), but found no significant differences. Many invertebrates are suitable subjects for this method. For example, we find that the bumblebee retina contains receptors peaking at 525 nm which have a sensitivity curve typical of an invertebrate green-receptor.

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The effect of reduced salinity on respiration and heart rate in the Calico crab, Ovalipes ocellatus. GEOFFREY F. BIRCHARD, LAWRENCE DROLET AND LINDA H. MANTEL.

The portunid crab *Ovalipes ocellatus* was found to be a stenohaline osmoconformer. Mature male crabs could be acclimated down to 60% salinity by small steps; mortality reached 100% below this salinity. Crabs were isosmotic to the medium at all salinities.

Oxygen consumption in crabs acclimated to 100% sea water was measured successively at 100, 80, 60, and 100% sea water with 0.5 hr acclimation periods between each salinity. Individuals showed a significant depression in oxygen consumption at reduced salinities. A marked increase in oxygen consumption, to a level greater than the initial control, was observed upon return to 100% sea water after exposure to hyposmotic conditions.

Oxygen consumption was also measured in animals acclimated to 60% sea water for 6–8 days. Oxygen consumption remained depressed in these crabs when measured at 60% sea water. Maximum oxygen consumption occurred in 100% sea water, indicating a lack of metabolic acclimation. Behavioral observations on crabs acclimated to 100 and 60% sea water indicated that part of the reduction in oxygen consumption was due to a decrease in locomotor activity at low salinity.

Heart rate was measured in crabs acclimated to 100% sea water, exposed acutely to 60% sea water, and then returned to full sea water. No significant difference in rate was observed between control values and those measured when crabs were subjected to hyposmotic stress for 1.5–2 hr.

When observed in shallow water in the field, *O. ocellatus* is usually buried in the substrate. When presented with a container of sand in the laboratory, the crab burrows immediately, usually leaving only its second antennae exposed. Oxygen consumption was measured in buried and exposed crabs. When buried, crabs showed a reduction in oxygen consumption of 30–70%. Preliminary experiments using buried and exposed crabs acutely exposed to 50% sea water indicate differential survival, in dilute salinity, with buried animals surviving longer. It is proposed that the substrate may afford a refuge for *O. ocellatus* during short-term hyposaline conditions within its environment.

This project was carried out as part of the Experimental Invertebrate Zoology Post-Course program.

Micrococcal DNAase studies on Spisula solidissima *chromatin structure.* MARK BOOTHBY, KELLY TATCHELL, AND K. E. VAN HOLDE.

S. solidissima oocytes have undergone neither of their meiotic divisions. Their intact germinal vesicles (GVs) were isolated by oocyte treatment with 1 M glycerol, 1 mM KPO_4 pH8, addition of an equal volume of 1% Triton X-100 (TX), 67 mM glycine, 13 mM 2-mercaptoethanol, 13 mM K-Tris-maleate pH 7.4, with or without 6.7 mM $MgCl_2$, and pelleting through 5% sucrose, 10 mM Tris pH 7.2 either with 0.75 mM $CaCl_2$ (STC) or with no $CaCl_2$. Nuclei from 8-cell stage embryos also were isolated by this method. Pellets were resuspended in STC for digestion. Sperm chromatin structure was studied in conventionally isolated nuclei and chromatin. All digestions used 125 μ/ml Worthington micrococcal DNAase (MN) and were quenched by EDTA addition to 5 mM after incubation at 37° C. Samples were pronase treated; GV and 8-cell stage embroys' nuclei were RNAase treated.

Sperm digestion time courses showed the sperm chromatin to be packaged as 140 base-pairs (bp) nucleosomes spaced on the average by 90 bp for a 230 bp repeat. The putative nucleosome, isolated from a chromatin digestion by sucrose density gradient sedimentation, sedimented at 11.3 S. GV digestion products fell into two rough series of approximate DNA sizes. The first series starts at 200 bp, the second at 300 bp; each then ascends in roughly 200 bp size increments. These data are consistent with oocyte chromatin packaging in nucleosomes, with a 200 bp repeat. This interpretation is supported by electron micrographs of GV chromatin which show clear nucleosome packaging. The second series suggests either paired nucleosome structures not separated by spacers or intra-nucleosome DNAase cutting, as well as normal cleavage in spacer regions, MN's preferred targets. In the latter case, unusually active DNAases endogenous to GVs may be responsible. Digestion products of 8-cell stage embryos' nuclei are most consistent with a 220 bp repeat and nucleosome packaging.

EM data on GV chromatin courtesy of Dr. Gwen Howze. This work was supported by PHS grant GM-00265; MB by NIH MSTP grant GM-07200; and KT by ACS grant NP-201.

Oxygen-hemoglobin relationships in the killifish, Fundulus heteroclitus. THOMAS A. BORGESE, JOHN HARRINGTON AND RONALD NAGEL.

Red cells of *Fundulus heteroclitus* contain four hemoglobins (I, II, III, and IV) which account for 13, 36, 36, and 15%, respectively, of the total hemoglobin pool. Ion-exchange chromatography of the neutralized TCA extracts indicate that ATP is the major organic phosphate at 2.20 μ moles per ml cells. Inorganic phosphate, AMP, ADP and GTP are present at concentrations of 4.91, 0.58, 1.10, and 0.52 μ moles/ml cells, respectively. For oxygen equilibrium studies, hemolysates were purified and equilibrated on sephadex G-25 columns with Tris-HCl buffer pH 8.3. The same buffer was used to equilibrate and elute the hemoglobins from DE-52 columns. The isolated components were subsequently passed through sephadex G-25 columns adjusted to pH 7.4 with 0.05 M Bis-Tris. All preparatory steps were done at 4° C. The oxygen equilibria of the isolated, purified hemoglobins were compared with identical samples supplemented with ATP in a nucleotide to hemoglobin ratio of 10:1. In every case the addition of ATP decreased the oxygen affinity nearly two-fold. Although each hemoglobin showed a marked increase in oxygen affinity with increase in pH, all seemed equally sensitive to pH changes over the range 7.4 to 8.5 at 15° C. The extent of hydrophilic interactions among the hemoglobin sub-units was examined by auto-oxidation experiments at 15° C in 0.1 M potassium phosphate buffer pH 7.4. Companion samples were supplemented with 0.5 M sodium chloride, and at recorded intervals the hemoglobin spectrum was obtained with a Cary

14 recording spectrophotometer from 500 to 700 nm. After two hours, potassium ferricyanide was added to convert the sample to methemoglobin. The difference between the two spectra at 540 nm was used to determine the rate of methemoglobin formation. Hemoglobin I was most sensitive to salt induced auto-oxidation with only 37% oxyhemoglobin remaining after two hours. This compares with 57%, 68% and 66% for hemoglobins II, III, and IV, respectively. It is concluded that: first ATP is the major organic phosphate in killifish red cells; secondly, killifish hemoglobins are equally sensitive to an ATP-induced decrease in oxygen affinity; thirdly, each hemoglobin shows a pH-dependent increase in oxygen affinity over the range 7.4 to 8.5; and fourthly, hemoglobin I has more critical hydrophilic interactions as judged by its increased sensitivity to auto-oxidation in the presence of 0.5 M sodium chloride.

Studies on cell populations from the marine sponge Microciona prolifera *separated by Ficoll gradients.* WERNER BURKART AND MAX M. BURGER.

Cell suspensions from dissociated *Microciona prolifera* contain several cell types. The most abundant cells present are choanocytes, archeocytes, gray cells and rhabdiferous cells. To separate the different cell types, the crude cell suspension containing 10^7 cells/ml in 8% Ficoll was layered on a Ficoll gradient ranging from 10% to 24% in 2% steps. The gradients were centrifuged 50 min at $60 \times g$, and 13 fractions were collected from the bottom. Fraction 12 (second from the top) contained up to 85% choanocytes and fractions 9 and 10 about 70% archeocytes. Fractions 3–7 contained most of the gray cells and the rhabdiferous cells plus archeocytes. Fractions 12 and 10 were run a second time on separate Ficoll gradients. The two resulting peak fractions contained more than 90% choanocytes and 75 to 80% archeocytes, respectively.

The gradient fractions were stained with Alcian Blue (AB), which in acid solution selectively stains acid mucopolysaccharides such as *Microciona* aggregation factor (MAF). Rhabdiferous cells and gray cells were heavily stained, while choanocytes and archeocytes only slightly stained. Chemically dissociated cells stained less than mechanically dissociated cells. Addition of MAF to the cells increased the staining.

Response of the different gradient fractions to MAF was tested in two different ways. First, soluble MAF promoted aggregation of the cells in all fractions. Secondly, MAF was covalently bound to Sepharose 4B beads. Binding of the cells of different gradient fractions to these beads in the presence of small amounts of MAF showed no differences. In all fractions about 95% or more of the cells bound to the beads. By visually examining the cells which remained single and those attached directly to the beads, there was no evidence that one distinct cell type binds preferentially to the MAF beads. From these results it is concluded that the main cell populations all contain at least either MAF or receptor for MAF on their surface.

Rhabdiferous cells were unstable in calcium magnesium free sea water. If temperature was increased, disintegration could be observed.

Supported by the Swiss National Science Foundation.

The regeneration of cilia of Arbacia punctulata *blastulae.* ROY BURNS.

The normal cilia of *Arbacia punctulata* blastulae have a mean length of 18 μm, while those of embryos animalized by treatment with 50 μg/ml trypsin for 12–18 hr are of variable lengths, approaching a maximum of 48 μm. On deciliation with hypertonic sea water, both normal and animalized cilia regenerate, but while the normal cilia regain their initial lengths within 120 minutes, the animalized cilia continue to grow for at least six hours. Detailed analysis of the regeneration of the animalized cilia indicates biphasic kinetics, with an initial elongation rate of 0.35 μm/min, followed by a period of continued growth at 0.05 μm/min until the maximum length is attained. Emetine (10 μM) inhibits cytoplasmic protein synthesis by at least 93% and slows the rate of elongation of normal and animalized cilia by approximately 25%. However, normal cilia regenerate to their initial lengths, indicating that protein synthesis is not required. By contrast, the regeneration of animalized cilia terminates at the end of the initial phase (14 μm), suggesting that the second, slower, phase reflects a requirement for *de novo* protein synthesis of a limiting component(s). When emetine-treated animalized embryos are deciliated for a second time, at the end of the initial growth phase, cilia regenerate again at the same initial rate but to only half the length (7 μm), indicating that the cessation of elongation is not due to exhaustion of a precursor pool. The results suggest that only about 50–60% of the precursor pool of the limiting component(s) is assembled into the organelle during elongation, and that the remainder is required as a cytoplasmic pool to maintain the equilibrium towards the assembled state.

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Community respiration rates of marine mud and sand sediments near Woods Hole, Massachusetts. COLLEEN M. CAVANAUGH AND BRUCE J. PETERSON.

Benthic respiration of mud and sand was measured and compared at two subtidal sites, Phinney's Harbor and the Northwest Gutter near Naushon Island, during spring and early summer, 1977. Intact sediment cores with the overlying water were obtained by divers and changes in oxygen concentration monitored in the laboratory for five to eight hours with oxygen electrodes. The least squares linear regression between rate of oxygen consumption and oxygen concentration was used to estimate the rate of oxygen uptake at oxygen saturation for the three to six replicate cores in each run. A control core filled with sea water was run to estimate respiration in the water column. Sediment respiration was corrected for plankton respiration in the overlying water. All benthic respiration rates are reported as mg $O_2/(m^2 \cdot hr) \pm$ the 95% confidence interval.

Respiration of the mud community at Phinney's Harbor and Northwest Gutter at 10° C was 67.5 ± 7.1 and 66.0 ± 8.7. The rates for the same mud communities at 16° C were 93.3 ± 13.4 and 111.1 ± 32.3. Respiration rates for the sand communities at Phinney's and Northwest Gutter at 12° C were 58.6 ± 23.1 and 25.3 ± 8.7 and at 18° C were 50.6 ± 3.7 and 50.8 ± 11.3.

Respiration rates of mud and sand were similar for a given sediment type and temperature with the exception of the sand cores run at 12° C. In all cases, respiration rates for mud were higher than those of sand. Mud sediments also had higher standing stocks of bacteria, benthic algae and invertebrates, as well as a higher percentage of organic matter.

Plankton respiration ranged from 40 to 90 mg $O_2/(m^3 \cdot hr)$ and water depth at the study sites ranged from 1 to 5 meters. Consequently, plankton and benthic respiration were of similar magnitudes at these shallow marine sites.

We thank John Teal and Michael Piotrowski for the use of the oxygen electrodes and recording equipment. This work was supported by the National Oceanic and Atmospheric Administration under grant 03-7-022-35133.

Morphological correlates of efferent circadian activity and light adaptation in the Limulus *lateral eye.* STEVEN C. CHAMBERLAIN AND ROBERT B. BARLOW, JR.

Morphological changes in the ommatidia of the dark adapted (DA) *Limulus* lateral eye occur on a day/night basis and appear to be controlled by efferent activity in the optic nerve trunk. At noon cone cells separate the rhabdome of a DA ommatidium from the base of the cuticular cone by nearly 30 μm forming an aperture (diameter 17 μm) surrounded by pigment cells. The rhabdomeral region is free of screening pigment and is elongated parallel to the optic axis of the cuticular cone. At midnight, during the period of efferent optic nerve activity, the cone cell region is reduced to less than 4 μm and the aperture is widened to over 40 μm. The rhabdome region is shortened by 40%, widened by 50%, and compressed against the base of the cuticular cone. Stimulating the efferent input to the eye by shocking the optic nerve at noon increases the ERG amplitude and transforms the structure of the ommatidia from the noon DA state to the midnight DA state. Thus, structural changes account for at least part of the circadian changes in the sensitivity of the *Limulus* lateral eye.

Ultrastructural changes in the rhabdome are also correlated with circadian efferent activity. The thickness of the rhabdomere in animals DA for 24 hr is 4.5 μm at midnight, 3 μm at noon and less than 2 μm at 4 PM. Upon the first exposure to sunlight after 24 hr of DA, vacuoles are formed which reduce the area of photoreceptive membrane in the rhabdome to at most 50% of the daytime DA level within 15 min. After 30 min of light exposure the rhabdome has been rebuilt and the cytoplasm adjacent to it is filled with multivesicular bodies which coalesce and move away from the rhabdome to the periphery of the retinular cells during the next 8 hr of light exposure. Control experiments suggest that the transient turnover seen at first light onset may not be a general property of light adaptation but rather a cyclic process analogous to the daily sloughing of rod outer discs. Time of day is thus an important parameter in morphological studies of the *Limulus* lateral eye.

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Voltage clamp studies of chemical driving force of sodium ions at nerve excitation. DONALD C. CHANG.

Voltage clamp studies of chemical driving forces of Na^+ ions in squid giant axon were performed to test the ionic theory of Hodgkin and Huxley and a model of nerve axon recently developed by this author. The basic assumptions of this model are: first, at the resting state, the mobile ions are distributed at quasi-equilibrium; secondly, the axoplasm behaves as an ion-exchanger; and thirdly, the axon cortex (*i.e.*, a highly structured layer of axoplasm attached to the membrane) can exist in more than one metastable state. The ion-selectivity changes at excitation as a result of transition between these states. This model predicts that, during excitation, the chemical driving force of Na^+ ions at the axon surface will change as a function of time. To test this model and the ionic theory (which maintains that the chemical driving force of Na^+ is a constant), the chemical driving force of Na^+ ions at the axon surface was measured by determining the instantaneous current-voltage relationship using a two-step voltage-clamp method. A double perfusion technique was also used to control the ionic concentrations. The experimental result shows that the chemical driving force of Na^+ ions varies as a function of time and voltage. At the resting state, the chemical potential of Na is approximately the same as the resting potential. The chemical potential gradually changes toward the Nernst potential when the axon is depolarized. When the depolarization is turned off, the chemical potential relaxes back to the resting value again. These observations support the present model over the ionic theory.

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Possible improvements in optical methods for monitoring membrane potential. L. B. COHEN, K. KAMINO, S. LESHER, C. H. WANG, A. S. WAGGONER AND A. GRINVALD.

Giant axons from the squid, *Loligo pealii*, were used in experiments trying to find better signals for optical monitoring of membrane potential. We were able to improve the signal-to-noise ratio obtained in birefringence experiments (dyes XVII, NK 2367, and several analogues) by a factor of five by using cut-on filters with wavelengths longer than 750 nm rather than 30 nm band-pass filters. The resulting signal-to-noise ratios were in the range 30–50. Dye bleaching and photodynamic damage are eliminated by using wavelengths outside the absorption band of the dye.

Dyes XVII and I were modified by replacing the negatively charged sulfonate with a positively charged quatenary ammonium group. Changing the sign of the charge did not affect the direction of the signal. In addition, the signal-to-noise ratios and wavelength dependences were similar for the oppositely charged pairs. Such pairs could be useful in ruling out artifacts from charge effects.

We have found a better fluorescence dye. A signal-to-noise ratio of 8 and a fractional change of 2×10^{-3} was obtained with an asymmetric pentamethin oxonol with 1,3, dibutyl barbituric acid (5) and 1,(p-sulfophenyl) 3-methyl, 5-pyrazolone (4) groups. The photodynamic damage with this dye was 30 times smaller than that found with dye I. Because of its large fractional change, we attempted to use a 50 mwatt helium-neon laser as a light source. We were able to reduce the noise in the laser output by a factor of about 50, stabilizing the output to one part in 10^4. However, the signal-to-noise ratio obtained in fluorescence experiments was only about 8, which is smaller by a factor of 3–5 than the expected improvement. This discrepancy may be due to spatial inhomogeneity in the noise in the laser output, and we are trying to overcome this difficulty.

Rapid visualization of the marginal band system in blood cells of marine species.

WILLIAM D. COHEN, IRIS NEMHAUSER, AND RICHARD JAEGER.

The marginal band (MB) is a continuous peripheral bundle of microtubules originally discovered in the elliptical, nucleated erythrocytes of nonmammalian vertebrates. Its probable role in generation and/or maintenance of cell ellipticity has not yet been elucidated. In living erythrocytes the MB is obscured by hemoglobin, but partial lysis in an appropriate medium renders it visible in phase contrast. A modified microtubule polymerization medium was employed containing Triton X-100 in a survey of six local marine fish species: the porgy (*Stenotomus chrysops*), killifish (*Fundulus heteroclitus*), cunner (*Tautogolabrus adspersus*), toadfish (*Opsanus tau*), dogfish (*Mustelus canis*), and skate (*Raja erinacea*). Erythrocytes of these species range from relatively small in the porgy (major axis approx. 11 μm) to large in the dogfish and skate (approx. 22 μm). MB thickness in semi-lysed cells shows a general positive correlation with cell size.

Semi-lysed fish erythrocytes consist of nucleus and MB interconnected by material nearly transparent in phase contrast. MBs of semilysed dogfish cells often twist, with time, into figure-8 shapes. In favorable views, trans-MB sheet-like material (TBM) can then be seen. Figure-8 MB forms may be the result of TBM shrinkage or contraction, with the MB accommodating to reduced surface area. The MB might then function as a flexible structural frame across which the TBM applies a tension, thus generating an ellipse.

The lytic medium was used to search for MBs in blood cell types which have received little attention, *i.e.*, in invertebrates. The MB system is apparently more widespread phylogenetically than previously suspected, occurring in the following species representing two phyla: *Golfingia gouldi* (Sipunculoidean worm), *Limulus polyphemus* (horseshoe crab), and the marine crabs *Cancer borealis, Carcinus maenas,* and *Libinia emarginata.* Except for *Golfingia,* the cells in question are not erythrocytes.

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Genetic similarities of wood-boring bivalves (Pholadidae and Teredinidae) based on comparisons of allozymes.

TIMOTHY J. COLE AND RUTH D. TURNER.

Deep-sea wood-borers (Xylophagainae; Pholadidae) converge strongly with shipworms (Teredinidae) in several features. They both have a wood-storing caecum, use wood for protection and for nutrition, have remarkably similar shell morphologies and similar modes of boring, and line their burrows with calcium [at least in one xylophagid genus (*Xyloredo*)].

We investigated relatedness among selected species within the families Pholadidae (Martesiinae: *Martesia striata;* Xylophagainae: *Xylophaga atlantica, Xylophaga* sp., *Xyloredo ingolfia*) and Teredinidae (*Lyrodus pedicellatus*) using allozymic characters revealed by horizontal starch-gel electrophoresis. Sample sizes ranged from 21 (*Xylophaga* sp.) to 31 (*Xyloredo ingolfia*). Twenty-two loci were resolved in *Lyrodus pedicellatus* and *Xylophaga* sp., 18 each in *Martesia striata* and *Xylophaga atlantica*, and 17 in *Xyloredo ingolfia*. Nei's index of genetic similarity was calculated from the data. This index is the normalized identity of genes per locus between compared species. Unity occurs when allelic frequencies are identical, while zero values indicate no common alleles.

Comparisons showed that species of Xylophagainae are much more genetically similar to each other (*Xylophaga atlantica vs. Xylophaga* sp.—0.487; *Xylophaga atlantica vs. Xyloredo ingolfia*—0.230; *Xylophaga* sp. *vs. Xyloredo ingolfia*—0.238) than either are to *M. striata* (0.107; 0.093; 0.059, respectively) or to *L. pedicellatus* (0.003; 0.019; 0.088, respectively).

The current phylogenetic position of the Xylophagainae is not affected by these results because on an electrophoretic basis members of this subfamily are no more closely related to *M. striata*, a representative of another wood-boring pholad line, than to *L. pedicellatus*, a shipworm. Interpretation of similarity values is difficult, however, due to a lack of comparable data from other marine invertebrates.

We thank the crew of the R/V ALBATROSS IV, National Marine Fisheries Service, Woods Hole, and of the DSRV ALVIN, Woods Hole Oceanographic Institution, for aid in obtaining specimens. This study was supported by ONR Contract N000–14–76–C–0281, NR 104–687 with Harvard University.

A study of chitin digestion by luminescent bacteria. YVONNE COLLINS, ALEX KEYNAN AND J. W. HASTINGS.

Although all known species of luminous bacteria produce extracellular chitinase and digest chitin, luminescent bacteria have not been reported from enrichment cultures with chitin. Our objectives were to investigate whether chitinolytic bacteria isolated by enrichment on chitin are luminescent and to study the chitin digesting activity of luminous bacteria. Optimal conditions for screening for chitinolytic bacteria were investigated. The best method found was an agar plate containing a ten-fold dilution of standard nutrients overlaid with sea water agar containing 1% purified colloidal chitin, which appeared turbid. Clearance of the turbidity during bacterial growth indicated chitin digestion, and a clear zone extending beyond colonies indicated production of a diffusible exoenzyme. Strains of all major groups of luminous bacteria were found to digest chitin, including *Photobacterium fischeri* (MJ-1), *P. phosphoreum* (WSU and NZ-1), *P. leiognathi* (721 and S-1), and *Beneckea harveyi* (MAV 392). Between 18° and 28° C digestion of chitin could be detected within four days. At 11° all strains of *Photobacterium* digested chitin, but required longer, between one and two weeks. Luminous bacteria readily give rise to "dark" variants, revertible to brights. Twenty "dark" variants were isolated and found to be positive for chitinolytic activity. Enrichment for luminous bacteria did occur in cultures with purified chitin in sea water. The number of bacteria increased in one week from 10^2 to 10^5, while luminescent bacteria, not detectable originally, increased to 2% after three days and 20% after six days. These experiments indicate that under these enrichment conditions luminescent bacteria have some advantage over other bacteria. In our experiments enrichment for luminous bacteria did not occur if, in addition to chitin, NH_4 was added. This might explain why previous investigators, who used chitin plus some nitrogen source, did not describe luminescent bacteria from their enrichment cultures.

Egg proteases of Arbacia punctulata: *evidence for a new elastase-like enzyme.* JOHN G. CSERNANSKY, GARY A. ROSMAN, ALBERT GROSSMAN, MORRIS ZIMMERMAN AND WALTER TROLL.

Trypsin-like enzymes secreted by the egg during fertilization have been described and assayed as TAME (tosyl-arginyl-methyl ester) esterase and protamine endopeptidase. To further characterize this activity, we employed a series of fluorogenic peptide substrates specific for trypsin, chymotrypsin, and elastase, with the general formula, 7-R-amido-4-methylcoumarin. The product of hydrolysis, 7-amino-4-methyl courmarin (AMC), was measured fluorometrically (excitation 380 nm, emission 460 nm) in 50 mM TES containing 10% DMSO at pH 7.5. The production of AMC was linear with time and enzyme concentration for all enzymes tested.

Fertilization product from *Arbacia* eggs fertilized with sperm contained protease activity against three peptide substrates, carbobenzoxy-Gly-Gly-Arg-AMC, Ala-Ala-Phe-AMC, and N-acetyl-Ala-Ala-Pro-Ala-AMC. These peptides are specific and sensitive substrates for trypsin, chymotrypsin, and elastase, respectively. The trypsin-like enzyme appeared in the sea water upon fertilization or activation by ionophore A23187 and was identical in substrate specificity and inhibitor profile to the previously described cortical granule protease. Antipain, leupeptin, soybean trypsin inhibitor (SBTI), and diisopropylfluorophosphate (DFP) inhibited this enzyme, whereas elastatinal had no effect. The enzyme was isolated from the fertilization product by precipitation at pH 4.0, and recovered in quantitative yield. The chymotrypsin-like enzyme was identified as an enzyme of the spermatozoa.

The elastase-like activity observed in the fertilization product was also present in sea water containing unfertilized eggs and over a period of 24 hours this activity markedly increased. The release of this enzyme may have been spontaneous or may have reflected release from disrupted eggs. The elastase-like activity was nondialysable, heat labile, and completely destroyed by treatment at pH 4.0 for five minutes. The enzyme was inhibited by antipain, elastatinal, and DFP, but not by leupeptin or SBTI. Eggs fertilized in the presence of 1.0 mM elastatinal exhibited normal fertilization, and subsequent development to plutei. Elastase-like activity could not be demonstrated in the perivisceral fluid of *Arbacia*, and homogenates of 12 hour embryos retained only 33% of the enzyme present in unfertilized eggs. Whether this elastase-like enzyme has a significant extracellular role in embryogenesis or whether its main function is intracellular, remains to be determined.

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Characterization of messenger ribonucleoprotein particles from the sea urchin Lytechinus pictus. CRAIG CUMMINS AND TIM HUNT.

Sea urchin eggs contain a store of active mRNA which is not translated until after fertilization. This mRNA is not associated with ribosomes, but is found associated with proteins in complexes ranging in size from 15–60 S probably according to the size of the mRNA. We wished to characterize these complexes in terms of their mRNA and protein content, with particular reference to the question of whether the proteins played a role in the prevention of translation of the mRNA prior to fertilization.

Eggs or 4-cell stage embryos were homogenized in 4 volumes of buffer containing 200 mM K acetate, 100 mM glycine, 50 mM NaCl, 50 mM HEPES, 5 mM EGTA,, 0.1 mM dithiothreitol pH 7.0. The 30 S from this extract was layered over 15–45% sucrose gradients in 200 mM KCl, 20 mM HEPES pH 7.2, 6 mM $MgCl_2$, 1 mM EDTA, and centrifuged for 12 hr at 25,000 rpm in the SW-27 rotor. Fractions were taken from this gradient corresponding to approximately 65 S, 50 S, 36 S, and 23 S. They were dialyzed overnight against 80% saturated $(NH_4)_2SO_4$, 50 mM Tris-Cl pH 7.5, 5 mM EDTA, 14 mM mercaptoethanol, harvested by centrifugation and taken up in and dialysed against 25 mM KCl, 10 mM NaCl, 10 mM Tris-Cl pH 7.5, 1 mM $MgCl_2$, 0.25 mM dithiothreitol.

These fractions were added to the reticulocyte messenger RNA-dependent protein synthesis system; they did not stimulate significant protein synthesis. However, the RNA extracted from these fractions with phenol : chloroform 1 : 1 was highly active, with the 50 S and 65 S fractions containing most of the mRNA. In contrast, equivalent fractions from embryos had at most 5% of the activity of the RNA from egg messenger ribonucleoprotein (mRNP) particles. These results are consistent with the hypothesis that after fertilization mRNA is mobilized into polysomes from the mRNA pool.

This work was supported by NIH training grant 07098 and NIH research grant HD-06574.

Mysterious ring-shaped particles from Arbacia *gametic fluid.* WILLIAM L. DENTLER.

Although many studies have characterized gametes of sea urchins, little is known about the fluid which is ejaculated with the gametes (gametic fluid). Specimens of *Arbacia punctulata* were induced to shed their gametes with KCl or electric shock; gametic fluid was separated from the gametes by low speed centrifugation and the gamete-free fluid was examined by negative stain electron microscopy and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In both male and female gametic fluids the major structural components observed were hollow ring-shaped particles. The rings were composed of a single closed chain of subunits and, occasionally, broken C-shaped rings were observed. In male gametic fluid the rings had an outer diameter of 24.1 ± 1 nm and a wall thickness of 5.3 nm. In addition to the rings, accordian-shaped structures with an outer diameter of 34.8 nm and of various lengths were observed in male but not in female gametic fluid. In female gametic fluid, the rings had an outer diameter of 28.7 ± 6 nm and a wall thickness of 5.9 nm. The female rings were composed of 11 prominent subunits; by contrast, the male ring subunits could rarely be clearly distinguished. Rings were pelleted from gametic fluid by centrifugation (100,000 × *g*, 1 hr) and were analysed by SDS-PAGE. In both sexes 5 major protein bands were observed. Of these, bands at 200,000, 100,000, 80,000, and 65,000 daltons co-migrated in both sexes. In rings pelleted from female but not male gametic fluid the 200,000 dalton component migrated as two closely spaced bands. In both sexes these high molecular weight proteins contained sugar residues, as shown by PAS staining. Although the ring fractions compose significant proportions of the gametic fluids (0.09mg/ml packed sperm; 0.14mg/ml packed eggs) they had no detectable effect on either sperm motility or fertilization. Their function remains, at present, unknown.

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Dark carbon uptake rate: its change with time as estimated with the ^{14}C method. CLAUDE W. DEPAMPHILIS AND PATRICIA D. SMITH.

The carbon uptake rate of a pond water sample was determined with a ^{14}C tracer for a series of 5-minute incubations at various intervals after the sample's transfer to darkness. The uptake rate for samples killed with $HgCl_2$ was also measured, the intent being to obtain an uptake rate representing all nonbiological carbon retention. This figure could not be interpreted in this way since the effect of the mercury treatment was to make the cells take up carbon more rapidly than did the live cells.

The rate of carbon uptake in the live samples decreased significantly during the 6-hour study period. Regression analyses show a significant fit (at 0.01 level) to the data when the natural logarithm of the mean value for the carbon uptake rate at a given time is plotted against the total time in darkness. A second run of this experiment failed to show any significant trend in uptake rate. This was possibly due to a much lower initial light intensity.

An exponential decline in the carbon uptake rate after the onset of darkness suggests that a portion of the total dark uptake of carbon is photosynthetic. The photosynthetic reduction of CO_2 in the dark is possible if an excess of $NADP_{red}$ and ATP exists at the end of a light period. If this is one mechanism by which carbon is being taken into the living cells, then there is justification for adding a portion of the dark uptake value in the ^{14}C production estimates, rather than subtracting all the dark uptake. The effect of such a change on oceanic primary production estimates may be large. A repetition of this experiment, using a photosynthetic inhibitor, is desired.

Induction of a morphological transformation in coelomocytes from Lytechinus *and* Strongylocentrotus. KENNETH T. EDDS.

The coelomic fluid of sea urchins will form a cellular clot when withdrawn from the test of the organism. This clot formation can be prevented in many species by the addition of the divalent cation chelator, EGTA, and the constituent cells separated on a sucrose gradient. One cell type, the petaloid coelomocyte, obtained from either *Lytechinus* or *Strongylocentrotus*, can be induced to reversibly transform to its filopodial form by an extract made from *Strongylocentrotus* egg acetone powder. The transformation-inducing factor is, therefore, not species specific in its ability to elicit the transformation. The factor is present in a low ionic strength (50 mM NaCl, 10 mM Tris-HCl, pH 7.8) extract of the egg acetone powder. The extract, at 1 mg/ml protein content, is applied to a suspension of petaloid cells with a resulting final concentration of *ca* 0.1 mg/ml. Within 4–5 min all petaloid cells have transformed to their filopodial form. Analysis of the extract on SDS-polyacrylamide gel electrophoresis reveals several polypeptides, the major ones being 200,000, 150,000, 101,000, and 32,000 daltons. It is presently unknown if any of these are related to the activity of the factor. Moreover, it has been determined that the active portion of the factor is heat stable, nondialyzable and, based on ultrafiltration data, greater than 100,000 daltons.

Supported by NIH grant GM-23351.

Amino acid stimulated ATPases exhibiting transport specificities in the intestine of the toadfish, Opsanus tau. A. FARMANFARMAIAN, MARK V. ROBINSON AND DEREK BARKALOW.

We have previously reported that the intestinal absorption of amino acids and sugars in teleosts, *in vivo*, involves carrier mediated processes capable of uphill transport. These carrier mechanisms require more than 30 mM Na in the immediate environment of the membrane. Energetically these processes do not require a lumen-to-cell gradient of Na. Net absorption can be nearly normal when the Na gradient is reversed under optimal stirring provided there is sufficient Na available to the membrane carriers. Similar data have been reported for mammals *in vivo*. We have suggested that the energy for this transport is derived from cellular ATP which is directly coupled to sugar and amino acid active transport mechanisms, as it is to the Na-pump on the basal side of the epithelial cells. In support of this view we have shown that when ATP is added to luminal solutions, it contramimics the role of cellular ATP and reduces glycine absorption by as much as 50%. Currently brush border membrane preparations from mucosal scrape of toadfish intestine have been shown to exhibit alanine and glycine dependent ATPase activity. This activity is considerably reduced in the basal lateral membrane of the same scrape. Addition of 50–80 mM L-Alanine resulted in 15–30% higher ATPase activity, while D-Alanine stimulation did not exceed 10%. This stereo-specificity is identical to that exhibited by the alanine transport mechanism. Similar experiments with glycine gave a 5–20% increase in ATPase activity of various brush border preparations. This stimulation was reduced in the basal lateral membranes. This amino acid-ATPase requires Na and Mg but is K independent. These ionic requirements and localization on the brush border further suggest that the amino acid-ATPase is one and the same protein as the binding-carrier protein of the neutral amino acid transport mechanism and thus energetically analogous to the Na-K-ATPase of the Na pump. The results briefly presented here are the strongest evidence available to date in support of the direct coupling of ATP to the brush border transport of amino acids.

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Decreased extracellular sodium prolongs recovery from adapting light in Limulus *ventral photoreceptors.* ALAN FEIN AND J. SHERWOOD CHARLTON.

The recovery from an adapting light was studied in both voltage clamped and unclamped photoreceptors. Replacing 75% to 100% of the sodium in the artificial sea water (ASW) that bathed the preparation with either lithium or choline prolonged the recovery from an adapting light. The sensitivity of the photoreceptor was monitored by a series of constant intensity test flashes. After an adapting light, the sensitivity of the photoreceptor recovered more slowly in the low sodium ASW than in the normal ASW. Also, in these low sodium solutions the response to the adapting light was followed by a prolonged depolarization in unclamped photoreceptors and by a prolonged inward current in clamped photoreceptors. The effects of low sodium ASW on the response to the adapting light and on the recovery of sensitivity following the adapting light were graded with the intensity of the adapting light. The above effects were nearly absent at low adapting light intensities.

When 75% to 100% of the Na in the ASW was replaced by Li, the prolonged depolarization following a bright adapting light was associated with an increased membrane potential noise. This increased noise was graded with the intensity of the adapting light being absent at low light intensities. Lowering the external Ca from 10 mM to 1 mM reversed the effects of replacing 75% to 90% of the Na by Li. These findings suggest that there may be a competitive action between Na and Ca at the photoreceptor membrane.

The role of the asters which mediate polar body formation in setting up localizations of developmental potential in the nemertine Cerebratulus lacteus. GARY FREEMAN.

The factors which specify gut formation in the *Cerebratulus* larvae are uniformly distributed in the unfertilized egg. These factors become localized in the vegetal region of the embryo between fertilization and the eight-cell stage. Ninety-six per cent of the cytoplasmic fragments from the animal hemisphere of unfertilized eggs produce a gut after the fragments are fertilized and allowed to develop, while only 60% of the animal hemisphere fragments develop into larvae that form a gut when they are produced after fertilization between second polar body formation and first cleavage.

The role of asters in localizing the factors which specify gut development has been studied by removing the aster-forming region that mediates polar body emission and by using metabolic inhibitors that prevent polar body formation. If the polar body forming region of the unfertilized egg is cut out, the egg is fertilized and the animal hemisphere is isolated at the time which corresponds to the stage between second polar body formation and first cleavage, 90% of the cases form a gut. Cytochalasin B (0.001 M) and urethane (0.09 M) inhibit polar body formation in *Cerebratulus;* both agents can be washed out and will allow development to proceed.

Urethane inhibits aster formation and causes formed asters to disappear, while cytochalasin B has no effect on aster formation. When urethane is used to suppress polar body formation and the animal hemisphere is isolated at the time which corresponds to the stage between second polar body formation and first cleavage, no progress can be detected in setting up localization of gut forming potential. If cytochalasin B is used to inhibit polar body formation in a comparable experiment, only 62% of the animal hemisphere fragments produce a gut; this indicates that there has been a normal localization of developmental potential.

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An effect of light on the hatching pattern of Limulus polyphemus. Kathleen French.

Limulus from eggs fertilized *in vitro* or collected in the field was reared in the laboratory with a 12L:12D illumination cycle. Individuals were then maintained under various light conditions and observed from before the pre-hatch molt until they hatched. Light conditions were: continuous light (CL); continuous darkness (CD); artificial 12L:12D cycle in phase with daylight (LD1); 12L:12D cycle 180° out of phase with daylight (LD2); and natural light from a north-facing window. The temperature was the same in experimental (LD1, LD2) and control groups.

Molting occurred with equal probability during the light and dark portions of the day. Hatching appeared to be equally likely in the two halves of the day in the CL and CD conditions. In contrast, animals exposed to light/dark cycles were most likely to hatch during the light portion of the cycle (for LD1: $n = 44$, $\chi_1^2 = 11.64$, $P < 0.001$; LD2: $n = 29$, $\chi_1^2 = 7.76$, $P < 0.01$; and NL: $n = 61$, $\chi_1^2 = 8.67$, $P < 0.01$). The 12-hour shift of the light-on portion of the cycle in LD2 did not change the result.

Sustained and vigorous activity is maintained during hatching and appears to aid the mechanical rupture of the deutovum. These results suggest that embryonic specimens of *Limulus* increase their activity during the light-on portion of a light/dark cycle. It appears, therefore, that very young specimens of *Limulus* respond to illumination differently from adults.

I thank Robert French for assistance in collecting the data. This work was supported by a grant from the Rowland Foundation to the Laboratory of Sensory Physiology.

Intracellular recording from terminal arborization of crayfish motor axon. Paul A. Fuchs.

Intracellular recording from terminal arborization of the claw opener motoraxons of the crayfish was accomplished by suspending the nerve on a small platform above the surface of the opener muscle. Microelectrodes filled with 3 M KCl, of 80 megohms resistance, were oriented along the long axis of the nerve, and penetrations of both the excitor and inhibitor were obtained. Resting potentials recorded in this manner ranged from 70 to 80 mV. Action potentials are 90 to 100 mV peak amplitude. Action potentials have depolarizing afterpotentials (DAPs) of 7 mV amplitude and 40 msec duration. The spike itself is 2.5 msec in duration. When trains of spikes are fired in either axon, succeeding spikes sum with the DAP of the previous spike, resulting in a net depolarization of 5 mV for a train at 40 Hz. Action potentials increase in duration 10 to 20% during trains at 40 Hz. This increase in duration is due to a decrease in the rate of repolarization.

One can observe hyperpolarizing IPSPs in the excitor axon in response to action potentials in the inhibitor. These IPSPs are 100 to 200 μV in amplitude and 60 msec in duration. If the inhibitor is fired repetitively, these IPSPs facilitate to 500 μV in amplitude. These hyperpolarizing IPSPs are assumed to be the intracellular sign of presynaptic inhibition. To date, the only observed effect of single IPSPs on the excitor action potential is a shunting of the DAP. There is no change in peak amplitude of the spike.

Preliminary experiments have shown that the DAP of the action potential in these axons may be due in part to a late inward calcium current. Both low external calcium and calcium blocking agents such as cobalt and cadmium reduce in size, or reverse, the DAP. Changes in external potassium have little effect on the DAP.

I thank the Grass Foundation for their support of this project.

Phosphorylation of Arbacia punctulata *sperm histones by cytoplasm from eggs and early embryos.* DAVID GARLING AND TIM HUNT.

The nuclei of *Arbacia* sperm contain highly distinctive histones H1 and H2B which are found in no other cells in this organism. It is believed that they are removed and substituted by egg histones prior to first cleavage. We thought to test the hypothesis that phosphorylation of these histones was involved in their removal from the sperm chromatin.

Histones from *Arbacia* sperm nuclei were extracted and incubated with cytoplasm from *Arbacia* eggs in the presence of added $\sigma-{}^{32}$P-ATP. Samples of these incubations were removed at intervals and analyzed by electrophoresis on 17.5% SDS-polyacrylamide gels, which were stained with coomassie blue, dried, and exposed to X-ray film. The sperm H1 and putative H2B histones were strongly and specifically phosphorylated; that is, essentially no labelling of H2A, H3 or H4 occurred. In controls without added histones, no labelling could be detected in the region of the histones.

The specificity of the *Arbacia* egg histone kinase(s) was tested by using sperm histones from *Lytechinus pictus* or calf thymus as substrates. In both cases H1 and H2B were strongly labelled, whereas the other histones were not. As far as we know, no other histone kinase has this particular specificity. Preliminary fractionation of the activity on DEAE cellulose suggests that there may be at least two enzymes involved in this system.

Cytoplasm extracted from eggs after 30 minutes of fertilization was also tested for the presence of the kinase activity and markedly lower levels of phosphorylation were found. More detailed studies of this apparent decline are necessary before its significance can be assessed.

This work was supported by NIH training grant HD-07098.

Cross-bridged microtubule arrays in embryonic cells of the horseshoe crab, Limulus polyphemus. DANIEL GIBSON.

"Fiber" cells underlying the dorsal skin of middle to late *Limulus* embryos were described by Wm. Patten in 1912 in *Evolution of the vertebrates and their kin* (P. Blakiston's Son & Co., Philadelphia, 486 pp.). The ultrastructure of the long coiled fibers within these 20 × 40 μm ovoid cells is shown by transmission electron microscopy to be bundles of up to 2500 parallel, cross-bridged microtubules. Tubule diameter is 225 Å; lumen diameter is 80 Å. Offset spacing of adjacent tubule rows causes the bundles to appear hexagonal in cross section, and creates intertubule distances of 75 Å and 125 Å, both of which are spanned by bridges. Spacing, tubule size, and degree of bridging appear unaffected by temperature of fixation (0° C *vs.* 20° C). Fixation employed one-hour immersions in 0.1 M cacodylate-buffered 2% glutaraldehyde and 1% osmium tetroxide, pH 7.6, adjusted to 1000 milliosmoles/kg with sucrose; 10 mM $CaCl_2$ was added to both fixatives.

Fiber cells do not persist as such into the larval stage. Patten believed that they metamorphosed into muscle, but it now seems more likely that they become tendinal cells, which typically contain microtubules in arthropods. This study revealed great numbers of aligned microtubules at dorsal muscle attachments in *Limulus* larvae; fiber cells are a likely source.

This work was supported by NIH grant R01-HL18267.

Nitrogen leaching from salt marsh grasses, Spartina alterniflora *and* Spartina patens. TERRI GOLDBERG AND IVAN VALIELA.

Nitrogen leaching from coastal marsh grasses, *Spartina alterniflora* and *Spartina patens* was measured by submerging leaves in glass tubes containing dilute low-nitrogen sea water. The results from samples taken during August show that more ammonium was released by plants in plots experimentally fertilized with nitrogen than by plants in untreated plots. The amount of nitrogen leached from both grasses increased from early to late August as the plants were flowering and setting seed. This marked increase agrees with an increase in export of dissolved inorganic nitrogen from Great Sippewisset marsh, the site of the present study, to the tidal water. The amount of leached ammonium was multiplied by the total biomass of *Spartina alterniflora* and *Spartina patens* to obtain an estimate of total ammonium released by the vegetation of the marsh. This total is approximately half the exported ammonium, and is therefore an important contribution to the nutrient export of salt marshes.

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Experimental transformation of muscle fiber properties in lobster. C. K. Govind, J. She, W. J. Costello and F. Lang.

The paired claws of adult lobsters, *Homarus americanus*, are dimorphic, consisting of a cutter and crusher claw. The distribution of these claws on right and left sides of the animal is random. The closer muscles are also dimorphic: the crusher muscle has all long sarcomere (>6 μm) slow fibers, while the cutter muscle has 65–75% short sarcomere (<6 μm) fast fibers with the remainder being slow fibers. The cutter closer muscle will twitch, closing the claw in 20 msec, while the crusher takes over 100 msec to close.

The claws and closer muscles of larval and early juvenile animals are symmetrical. The claws are cutter-like in appearance and both closer muscles contain 20–30% fast muscle fibers during the first two juvenile stages. During stage 5, one claw becomes established as a cutter and during the next stage it will have over 50% fast muscle fibers. The other claw will slowly lose the fast muscle fibers until it contains all long sarcomere slow fibers at stage 12–16.

This typical development can be influenced by environmental factors. Normally, the lobsters are reared in plastic trays which contain some crushed oyster shell in the bottom. If the crushed oyster shell is omitted, 20–30% of the animals will have two cutter claws: one closer muscle has about 90% fast fibers, while the other has 50–60% fast fibers, even as late as stage 15. Clearly the differentiation of muscle fiber properties in the lobster claw may be experimentally manipulated as is the case with vertebrate muscle. Furthermore, if the animals are raised in smooth bottom containers during stage 4 and 5, then switched to control conditions, some animals will have double cutter claws. Animals raised under control conditions during stage 4 and 5, then switched to smooth bottoms, do not have two cutter claws. Thus claw type appears to become fixed during stages 4–5.

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Acid rain, zooplankton fecal pellets and the global carbon budget. Charles A. S. Hall, Gil RoWe, John H. Ryther, and George M. Woodwell.

Some five to seven billion metric tons (BMT) of carbon dioxide are produced each year from fossil fuel burning and forest cutting. Of this, one to several BMT are unaccounted for in the increases in carbon dioxide observed in the atmosphere and dissolved in the sea. Any additional sink for this "missing" carbon must be large-scale, increasing and not balanced by feedbacks. Biotic processes of the sea could account for this "missing" carbon.

One billion metric tons of carbon divided by the area of the sea means that about 2.8 g (new) C must be removed in some way from the surface waters of the sea each year, an amount equal to about 4% of the primary production of the open sea. The proposed causal train is: increased nitrogen in rainwater → increased summertime primary production in stratified, nitrogen-limited, nutrient-poor oceanic waters → increased zooplankton feeding rates → increased zooplankton fecal pellet production → increased sequestering of carbon below the permanent thermocline *via* fecal pellet sinking. There is no doubt that this process is working to some extent, but are the rates sufficient to increase the zooplankton fecal pellet production by 2.8 $g/(m^2 \cdot yr)$? About 100 to 200 mg N/m^2 enter the sea surface each year in rain (an amount equal to all the dissolved nitrate in the upper 100 m of stratified Sargasso Sea water; both quantities are small compared to the amount annually incorporated as primary production). Analysis of the limited data available indicate that the nitrogen concentration of rain has approximately doubled in recent decades. The increase is caused by fixation of nitrogen during fossil fuel burning, which, with increased sulfur emission, causes acid rain. Assuming a Redfield ratio (C : N) for phytoplankton of 7 : 1 and a linear relation between N added and C removed, this new source of N falling on the sea could remove directly 0.7 to 1.4 g C/m^2, or 25–50% of the carbon "missing" from our knowledge of the carbon budget, by passing it across the permanent thermocline into the deep sea. This analysis can be compared to the few measurements of fecal pellet deposition in the open ocean, showing that at least 2 to 7 g $C/(m^2 \cdot yr)$ are being passed through the thermocline.

surements of fecal pellet deposition in the open ocean, showing that at least 2 to 7 g $C/(m^2 \cdot yr)$ are being passed through the thermocline.

Discrete wave frequency increases after bright illumination in Limulus *ventral photoreceptors.* Menachem Hanani and Alan Fein.

Discrete potential waves (bumps) have been recorded from several invertebrate photoreceptors. The available evidence indicates that these discrete waves occur when the photoreceptor absorbs a single quantum of light; hence, they have been termed "quantum bumps". Bumps have also been observed to occur spontaneously in the dark. The amplitude of both types of bumps is diminished after the cell is exposed to an adapting light. The effects of adapting lights on the bumps of *Limulus* ventral photoreceptors were studied on both voltage clamped and unclamped photoreceptors.

Reported here is the rather unexpected finding that in the dark after a bright adapting light there is a large increase in the frequency of the bumps. Both the increase in the frequency and the duration of the increase are graded with the intensity of the adapting light over a range of at least 2 log units. At its peak the rate of the bumps can increase by over a hundred-fold, compared to the rate in the dark. The recovery has a time course of several minutes. Changes in the sensitivity of the cell to light after a flash that increased the bump frequency were also measured. An increased frequency could be observed while the cell was still in a state of lowered sensitivity. Lowering the temperature from 20° C to 13° C decreased the frequency of the spontaneous bumps. A bright adapting flash increased the bump frequency but to a lesser degree than at 20° C. An increase of the temperature to 26° C had the opposite effect. These experiments suggest that the bumps that were observed after bright light are more similar to spontaneous bumps than to those that occur during light, although they themselves are somehow caused by light.

Lateral line and ocular-associated structures in the marine teleost, Stenotomus chrysops. Clifford V. Harding, Stanley Susan and S. Gregory Smith.

During the course of a study on the scanning electron microscopy (SEM) of corneal epithelial cell surfaces, a series of spine-like projections, each with a 'basal knob', were found at the corneal periphery, near the pit openings on the head, and near the lateral line. Epithelial cells, which entirely cover each projection, are continuous with the surrounding surface epithelium. Thin longitudinal sections for transmission electron microscopy show that each projection has a single large 'core' cell, covered with a monolayer of epithelium. The core cell has numerous densely-packed microtubules and microfilaments. The microtubules are more concentrated distal to the core cell nucleus, and the microfilaments more concentrated proximally. Mitochondria are distributed along the length of the core cell, with a high concentration at the base adjacent to the numerous microfilaments. The core cell has long interdigitations into the surrounding epithelial cells. Possible nerve connections remain to be established. Observations on the living projections can be easily made with Nomarski optics, utilizing thin slices of skin from the head region. The overall structure of the projection corresponds to that seen with SEM. However, in addition, the core cell could be seen and distinguished from the covering epithelial cells. Surface ridge patterns on the epithelium could also be detected. There is, therefore, no apparent loss or gross modification of the tissue during the preparation procedures for EM. Also, when fluid is forced through the pit opening there can be a resultant passive bending of the projection at the basal knob.

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A chymotrypsin-like enzyme present in Arbacia punctulata *spermatozoa.* Emily S. Harris, Sarah J. Fox, John G. Csernansky, Morris Zimmerman and Walter Troll.

Protease activity is released into the sea water during sea urchin fertilization. Previous studies have described a trypsin-like enzyme associated with cortical granule release in the egg.

Using specific fluorometric substrates and measuring the hydrolytic release of 7-amino-4-methyl courmarin (AMC), (excitation 380 nm, emission 460 nm), we noted chymotrypsin and elastase-like activity in the fertilization product in addition to the trypsin-like activity. The elastase activity is discussed in a separate communication. The chymotrypsin-like enzyme appeared to originate completely from sperm, since the activity was absent following activation of the eggs with calcium ionophore A23187. Moreover, only chymotrypsin-like activity was found in *Arbacia* spermatozoa. The enzyme activity was found in whole sperm, extracted by freeze-thawing and recovered in a 100,000 × *g* supernatant. The activity was characteristic of chymotrypsin activity in three aspects. First, it hydrolysed Ala-Ala-Phe-AMC. This substrate is not hydrolysed by trypsin or elastase. Secondly, as with chymotrypsin, the activity was inhibited by diisopropyl fluorophosphate and chymostatin, but not by antipain, leupeptin or tosyllysine chloromethyl ketone. Thirdly, the spermatozoa enzyme failed to hydrolyse the trypsin substrates tosyl-arginine-methyl ester and carbobenzoxy Gly-Gly Arg-AMC, and the elastase substrate N-acetyl-Ala-Ala-Pro-Ala-AMC. Activity in 1.0 ml dry sperm was equivalent to 100 ng chymotrypsin. Addition of calcium ionophore or jelly coat material from *Arbacia* eggs did not increase activity suggesting that there was no enzyme activation following shedding. The function of this enzyme may be to provide entry of the sperm into the egg.

Supported by grants from NIH.

Protein synthesis in the mullet in vivo. Audrey E. V. Haschemeyer and Michael A. K. Smith.

Striped mullet, *Mugil cephalus*, 200–300 g body weight, were captured in June, 1977, in estuaries in the region of Georgetown, South Carolina. Polypeptide chain elongation rate in liver protein synthesis was determined in anesthetized fish *in vivo* at 25° C by analysis of amino acid incorporation on ribosomes and in completed proteins at short times after hepatic portal vein injection. Average polypeptide chain assembly time was 3.0 ± 0.5 min, corresponding to an elongation rate of 2.3 ± 0.4 amino acid residues/sec. Ribosome concentration based on liver RNA analysis was 1.75 nmole/g liver. These data indicate a maximal protein synthetic rate of 40 mg/(g·day) in mullet liver at 25° C. Total protein synthesis was also determined from ^{14}C-leucine incorporation rate at 1–5 min after injection and liver free leucine concentration (0.53 ± 0.13 μmole/g). The result was 0.27 μmole leucine/(g·min) or about 45 mg protein/(g·day). After subtraction of plasma protein synthesis (24% of total), the synthesis rate for intrahepatic proteins, expressed as fraction of liver protein replaced per day (k_s), was 0.22. Whole body protein synthesis in mullet was studied by constant infusion of ^{14}C-tyrosine over a 4-hr period. Plasma free radioactivity rapidly reached steady state (exponential time constant $\lambda_p = 25$/day). Analysis of free pool radioactivity and incorporation into protein in terms of the constant infusion equations of Waterlow and Stephens yielded synthetic rates (k_s) of 0.21 for gill and 0.0065 for muscle. Determination of specific radioactivities of free and protein-bound L-tyrosine by fluorometric assay of tyramine is required for further analysis of these data.

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Olfactory discrimination of male and female conspecifics in the bullhead catfish, Ictalurus nebulosus. Pamela Herbert and Jelle Atema.

Bullhead catfish were trained by punishment and reward to discriminate between tank water from two equal sized donor fish, one male and one female. Several formal test series consisting of 10 positive and 10 negative trials presented at random were given and the fish's responses recorded. Four out of six fish trained showed discrimination although only one of them did so reliably enough for testing and subsequent nose plugging. This fish was able to properly discriminate male *versus* female odor in 18/20 trials. The fish's nose was then blocked with a cotton and vaseline plug and testing continued. In post-plug tests the fish no longer gave its learned response and therefore showed no discrimination. Such data has previously been interpreted as meaning that olfaction is essential for conspecific recognition. However, after nose plugging the fish did show evidence of sensing the stimulus (wiggling, pushing its head up to the stimulus introduction tube), probably through the taste buds covering the animal's skin. The fish gave no response to control stimuli of its own tank water. In the catfish both olfaction and taste are well developed senses with anatomically distinct connections to the brain. We therefore propose an alternative explanation for our data. Abolishment of the learned response with loss of olfaction implies that olfaction was used in the learning process and that taste could not immediately take over this function. It is not yet clear as to whether or not taste can be used for learning if olfaction is blocked before training.

This work was supported by NSF-URP Grant #4653-5.

Unique structural properties of surf clam tropomyosin. CHRISTINE L. HOWE.

Tropomyosins were prepared from Bailey powders of surf clam (*Spisula solidissima*) adductor muscle and rabbit back and leg muscle. Both tropomyosins had molecular weights of 35,000 on SDS gels. Clam tropomyosin migrated as one band at the same rate as the faster of the two rabbit chains. Isoelectric points determined on focusing gels were approximately pH 5. Tropomyosin-magnesium paracrystals of rabbit formed at pH 7.5 had the usual 390 Å periodicity, but clam paracrystals showed a shorter repeat period of 132 Å. This difference persisted when paracrystals were formed at pH 6.5 and 8.5. No hybrid periodicities were found in paracrystals formed from a solution of both clam and rabbit tropomyosins. Trypsin and chymotrypsin treatment for ten minutes at room temperature with protease : tropomyosin ratio of 1 : 500 had no effect on the formation of clam paracrystals. Rabbit tropomyosin paracrystals formed after these treatments were mostly amorphous, but a small number of paracrystals showed a 40–45 Å repeat period. Often, the alignment of these bands was offset so that a "rat-tail file" appearance was seen. Tropomyosin samples were carboxymethylated using guanidine HCl and iodoacetamide, dansylated, purified on a preparative SDS gel, and digested with trypsin for peptide mapping. After chromatography with 30% ammonium hydroxide, 70% n-propanol and electrophoresis with a pyridine-acetic acid buffer at pH 6.5, the maps were sprayed with fluorescamine and compared. Twenty-three peptides were the same in both tropomyosins. Clam tropomyosin had 19 unique peptides, and rabbit had 12 peptides not evident in the clam preparation. Thus, the difference in paracrystal structure between *Spisula* and rabbit tropomyosins is supported by differences in the primary amino acid sequence as reflected in the peptide maps.

Supported by NIH training grant GM-00265.

Electron microscopic detection of putative replication sites in chromatin from Arbacia *embryos.* G. B. HOWZE AND K. E. VAN HOLDE.

Preliminary electron microscope studies of replicating chromatin from nuclei of *Arbacia* embryos have detected loop-like structures in the chromatin fibers which resemble the "eye forms" which have been visualized in electron micrographs of replicating DNA. The tentative conclusion is that these chromatin loops are equivalent to "eye forms" and are indicators of the sites of DNA replication in chromatin.

Although only a few "chromatin eye forms" have been clearly photographed, interesting variations from the "eye forms" of free DNA have been noted. First, the thickness of the arms is greater than expected for free DNA. Secondly, a curious asymmetry in the lengths and thicknesses of the two arms of the loop has been observed. The thicker arm is also shorter, suggesting early compacting of DNA. Finally, large particles, not part of the linear structure of the fiber, have been seen associated with the thinner and longer fiber. Additional studies will be necessary to determine if the loops are the chromatin equivalent of "eye forms", and whether the structural differences listed above are standard components of "chromatin eye forms."

Radial propagation of positional signals for retinotectal patterns in Xenopus. R. K. HUNT AND CHARLES IDE.

During development, *Xenopus* retinal ganglion cells undergo position-dependent differentiation, acquiring the locus specificities that enable their axons to assemble patterned connections in the midbrain (retinotectal map). Little is known about how nascent ganglion cells (added to the retinal periphery over the many weeks of larval growth) receive positional information, but the spatial plan is fixed during a critical period of axis determination at (tail-bud) stages 28–31. We have exchanged small bits of tissue (embracing as few as 15–30 cells at the nasal, temporal or ventral pole of the stage 32–35 eye-bud) between two marker mutants: 1-nucleolate heterozygotes for the Oxford marker and homozygotes of the albino mutation (alb^p). Such chimeric eyes can be reconstructed by pigmentation and (at the single-cell level in retina) by nucleolar counts (since wild-type and albino cells have two nucleloi), after metamorphosis following electrophysiologic analysis of the retinotectal map. Nasal or temporal pole grafts (orthotopic or into each other's position) grow out contiguous 'pie-slice' polyclones subtending a nearly constant degree of visual angle from optic disc to lens. Ventral polyclones are similar, but they flare markedly from limbus to ciliary margin, indicating late larval overgrowth programmed into ventral cells very early and thereafter independent of their (implantation) site in the eye. Retinotectal maps are normal following orthotopic grafts. They usually show 'pie-slice' duplications, matching the location of the polyclone and retaining its axial polarities, in heterotopic grafts. Rarely, when polarity of the map-slice is absent or changed to match the host site, the loss or change involves one (anteroposterior, dorsoventral) axis. We infer that growth is radial, and positional signaling entails local radial propagation of Cartesian information.

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Studies on the activation of protein synthesis in the sea urchin embryo. HOWARD JACOBS AND TIM HUNT.

A cell-free protein synthesizing system was prepared from 4–8 cell embryos of *Lytechinus pictus*. Under optimal conditions, the initial rate of incorporation of labelled methionine represented about 10% of the protein synthesis rate *in vivo*. Extracts prepared identically from unfertilized eggs or immediately after fertilization membranes had appeared, had negligible protein synthesizing activity. Extracts prepared 5 minutes after fertilization showed 50% of the activity of the 4–8 cell extract. Methionine incorporation was inhibited by pancreatic ribonuclease and inhibitors of chain elongation (puromycin, emetine, cycloheximide). Chloramphenicol at 10^{-5} M had no effect. Initiation inhibitors gave ambiguous results: heparin caused no inhibition, aurintricarboxylic acid and edeine inhibited at unusually high doses, but edeine affected also the initial rate. By following the time-course of acid precipitable counts in the soluble and pelletable fractions, the average translation time was apparently about 15 minutes. After preincubation with puromycin, gel filtration through Sephadex G50 and reincubation with labelled methionine, sucrose density gradient analysis revealed the re-appearance of counts in polysomes. More than one interpretation of these results is possible, and it remains uncertain whether initiation is occurring. Homologous and heterologous (globin, TMV) mRNAs with substantial template activity in the message-dependent reticulocyte lysate, stimulated neither egg nor embryo extracts. Analysis of products by SDS polyacrylamide gel electrophoresis and autoradiography showed that the heterologous messages were not translated, the significance of which depends on establishing whether initiation is taking place. Experiments in which egg extract was mixed with embryo extract or reticulocyte lysate, or using systems reconstituted from post-ribosomal supernatants and Sepharose 6B gel filtered 'ribosomes', indicated the absence of a cytosolic inhibitor in the egg and failed to detect an activator in the embryo cytoplasm.

This work was supported by NIH training grant HD-07098.

Studies of the binding of Microciona prolifera *aggregation factor to dissociated cells.* JAMES E. JUMBLATT AND MAX M. BURGER.

Cells dissociated from some marine sponges have been shown by Humphreys and Moscona to reaggregate species-specifically in response to aggregation factors (AF) isolated from cell surfaces. AF from *Microciona prolifera* is a large (21×10^6 dalton) protein-polysaccharide containing approximately 40% carbohydrate. The binding of radioiodinated *Microciona* AF to cells was studied in order to elucidate some quantitative aspects of AF-cell interaction and the chemical basis of AF specificity.

Microciona AF was purified by the procedure of Humphreys, labeled with iodine-125 by the chloramine-T technique, and further separated by sucrose-gradient velocity sedimentation. The gradient peak fractions containing high amounts of radioactivity, aggregation-promoting and cell-binding activity, were pooled. No detectable loss of activity occurred as a result of these procedures. The radioiodinated AF had a specific activity of approx. 800 units/mg protein, and a specific radioactivity of 6.58×10^5 cpm/microgram.

AF binding was studied under the standard conditions of the aggregation assay: 10^7 cells/ml in artificial sea water, 20 minutes on a rotary shaker at room temperature. Except where indicated, AF was incubated with cells at the minimal concentration needed for aggregation (1 unit/ml). The following results were obtained. First, AF binding to cells was not saturable at high AF concentrations, probably because of the concentration- and cation-dependent self association between AF molecules. Secondly, at low AF concentrations (1 unit/ml or less), 300–500 molecules of AF were bound per cell after 20 minutes of incubation. Chemically and mechanically dissociated *Microciona* cells (either live or fixed with 1% glutaraldehyde) bound similar amounts of AF. However, fixed *Haliclona oculata* cells bound less than 50 molecules of *Microciona* AF per cell, thus demonstrating that the specificity of the AF extends to the level of binding. Thirdly, AF binding to cells (unlike AF-promoted cell aggregation) was not inhibited at low calcium concentrations (2 mM), or by pretreatment of the AF with 10 mM EDTA. However, AF binding to *Microciona* cells was strongly inhibited by soluble *baseplate*, a receptor-like surface component isolated from *Microciona* cells.

Supported by the Swiss National Science Foundation.

Calcium transients during early development in echinoderms and teleosts. DANIEL P. KIEHART, GEO. T. REYNOLDS AND A. EISEN.

To visualize increased cytoplasmic levels of free Ca^{++} during various processes in early development, eggs from the sea urchin *Lytechinus variegatus* and the teleost *Oryzias latipes* (Medaka), pre-microinjected with the Ca^{++}-sensitive luminescent protein aequorin, were observed during fertilization, mitosis, and cleavage using a microscope image intensification system. The light from the image intensifier (gain *ca.* 10^6) was observed directly and recorded on Polaroid 3000 and 10000 film. While luminescence was observed during fertilization, none was observed during mitosis or cleavage. Single *Lytechinus* eggs gave sufficient Ca^{++}-aequorin luminescence to permit a sequence of photographs during the activation process. The time resolution was limited to *ca* 5 sec. No wave of light was observed to traverse the eggs. Instead, the entire surface was emitting light within 7 sec of fertilization and continued to luminesce for longer than 60 sec. This is in contrast to the wave of Ca^{++} release observed in Medaka (Gilkey *et al.*, 1977, *Biophys. J.*, **17**: 277a). The threshold for detectability of Ca^{++}-aequorin luminescence in artificial sea urchin "cytoplasms" containing physiological levels of Mg^{++} was *ca.* 5–10 micromolar Ca^{++}. Therefore, during fertilization in sea urchins the Ca^{++} concentration increases to greater than 5 micromolar, but during mitosis and cleavage, no increase in free Ca^{++} above 5 micromolar is observed.

It takes *ca.* 10–20 sec for the wave of cortical vesicle breakdown to traverse the sea urchin egg, yet the entire surface of the egg was luminescing in less than 7 sec. It is concluded that while Ca^{++} may be necessary for the cortical reaction, its increase in concentration alone is not sufficient to cause cortical vesicle breakdown.

The cooperation of Dr. O. Shimomura, who supplied the aequorin, is gratefully acknowledged. This work was supported by NIH HD00030, NIH PHS GM-23475, NSFBMS 75-00473, ERDA Division of Biomedical and Environmental Research Contract Y-76-S-3120, and NIH MSTP GM-07170.

Light intensity effects on polyp size: studies with the stone coral Astrangia danae. JOHN R. KREZOSKI.

Polyp lengths of the ahermatypic Scleractinia *Astrangia danae* were measured at varying light intensities in a laboratory environment. Ten polyps from three individual colonies were photographed at eleven different light intensities after the polyps equilibrated at each intensity for one hour. Illumination from two 40 watt incandescent bulbs, controlled by a variable transformer, provided 0 to 17% of natural ambient light available to *A. danae* at 5 m depth during maximum insolation on a clear day.

Polyp lengths of *A. danae* increased linearly with increasing light intensity for those individuals which contained symbiotic zooxanthellae ($r^2 = 0.78$, $P < 0.05$), while those without zooxanthellae demonstrated no significant correlation with light intensity.

Transmitter release at identified frog denervated neuromuscular junctions. M. E. Kriebel, R. B. Hanna and G. D. Pappas.

The sartorius nerve was removed in small green frogs (*Rana clamitans*), and the time course of changes in the fine structure of nerve terminals and Schwann cells were followed as well as spontaneous potentials (MEPPs). The sartorius muscle was removed and placed in a small bath and small hooks were used to stretch the muscle so that edge muscle cells could be visualized with a compound microscope. The edge muscles were recorded from with micropipettes and the muscle fixed in place with glutaraldehyde so that studied cells could be identified with the electron microscope. MEPPs from nerve quanta ceased after 24 hours at which time the nerve terminal appeared dead. A few hours previous to this terminal stage of nerve function, synaptic vesicles were found to be clumped, and large bursts of MEPPs were recorded. From day 2 to 3 after nerve section, no MEPPs were recorded, and this stage has been termed the quiescent period which is terminated by the reappearance of MEPPs, resulting from quanta released from the Schwann cell. The Schwann cell made close contact with the postjunctional folds of the muscle as the nerve deteriorated. The frequency of Schwann cell MEPPs varied from a few/min to 100/min, and they formed a mode (about 200 μV) just out of the noise. The origin of Schwann cell quanta is not obvious since few, if any, vesicles were found in the Schwann cell, even in those cells generating 100 MEPPs/min.

Differential surface coat staining of snail hemocytes interacting with trematode parasites. Paul L. Krupa and Larry M. Lewis.

Previous work with electron microscopy has revealed that, in digestive glands of *Bulinus guernei* snails infected with *Schistosoma haematobium* daughter sporocysts and cercariae, hemocytes called granulocytes interact with sporocyst microvilli by engaging in contacts and other presumably preliminary activities leading to encapsulation of the parasites. In the present work comparable host and parasite tissues were stained with colloidal lanthanum hydroxide and ruthenium red (RR) and processed for electron microscopy.

Of particular interest is the striking variability and specificity of lanthanum and RR localization along the external surface of granulocytes. When found relatively isolated from sporocysts, granulocytes are roughly circular and oval in profile; their surface coat stains uniformly along the entire cell periphery. In contrast, when found next to or in apparent contact with sporocyst microvilli, granulocytes are found extended with their long axis parallel to the sporocyst surface; their glycocalyx stains intensely only along the surface distal to the sporocyst tegument. The glycocalyx of extended granulocytes proximal to the sporocyst tegument, at the granulocyte-parasite interface, stains sparsely or not at all.

Lanthanum and RR stain extracellular acid mucopolysaccharides and probably detect binding sites for polyvalent cations. Thus the above differences in cell surface coat staining are interpreted as reflecting real structural heterogeneity of granulocytes when they interact with parasite surfaces.

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Veratridine and internally perfused voltage clamped squid axons. D. Landowne and V. Scruggs.

Veratridine is a depolarizing agent which acts by producing a tetrodotoxin sensitive sodium permeability in excitable membranes. Using standard voltage clamp techniques, a brief depolarization is followed by a prolonged 'tail' of inward current when veratridine is included in the perfusion fluid. The amplitude of the tail increases with the amplitude or duration of the brief depolarization. At 11° C the tail decays with an 0.2 sec time constant. Repetitive pulsing results in summation of the tail currents and a concomitant decline in the amplitude of the fast sodium currents. Replacing external sodium with choline or Tris, adding tetrodotoxin externally, or adding Zn^{++} ions internally, all result in the absence of the veratridine tail. Internal treatment with pronase to remove sodium inactivation does not block the effect. These experiments suggest that the veraridine-treated membrane functions with the same sodium channels which produce the rapid sodium currents underlying the nerve impulse. If these channels are present and openable, veratridine is effective. The appearance of veratridine tails is associated with the disappearance of fast channels.

When external sodium was replaced by lithium the veratridine tail currents were reduced by about one half although the rapid currents changed very little.

Histochemical demonstration of trophic influences in lobster neuromuscular systems. F. Lang, W. J. Costello, M. Ogonowski and B. Roehrig.

The biochemical properties of lobster claw closer muscles were studied using two histochemical techniques. These muscles are of interest because like the claws, the closer muscles are asymmetric. The crusher claw closer muscle is composed entirely of long sarcomere claw muscle fibers, while the cutter claw closer muscle has 65–75% short sarcomere fast fibers, the remainder being slow. However, both closer muscles receive only two homologous motor axons, a "fast" and a "slow".

When stained for NADH. the cutter claw closer muscle behaves as expected: slow muscle has high oxidative capacity, while fast muscle has low oxidative capacity. The crusher claw closer muscle, however, stains according to the pattern of innervation of the muscle fibers. All muscle fibers have long sarcomeres, but the highest oxidative capacity is found in fibers which receive only the slow motor axon. Those fibers which are innervated primarily by the fast axon have lower oxidative capacity, while the fibers which receive both axons are intermediate in their staining properties.

When stained for ATPase, some of the results again suggest a trophic influence. In the cutter claw closer muscle, the slow fibers stain darkly, in a manner similar to the slow opener muscle fibers. The fast fibers stain more lightly. Thus the staining is apparently "reversed" as is observed under certain conditions in vertebrate preparations. In the slow crusher claw closer, most of the muscle fibers stain darkly. However, a band of muscle fibers in the medial region stains lightly, much like fast muscle, even though they have been characterized as long sarcomere slow fibers. This pattern of staining does not appear to be related to the distribution of motor axons.

Thus, it is clear that these lobster muscle fibers do not have a homogenous complement of physiological and biochemical properties which can be determined by sarcomere length. Rather certain muscle fiber properties appear to be matched to the pattern of motor neuron innervation, or to other as yet unknown factors.

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Assembly characteristics of dogfish brain tubulin. George M. Langford.

Dogfish brain tubulin was extracted and purified in 0.1 M 2(N-morpholino) ethanesulfonic acid (MES), pH 6.6, 0.5 mM $MgCl_2$, 1.0 mM ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), and 1.0 mM guanosine triphosphate (GTP) by a temperature dependent assembly-dissassembly procedure. After two cycles of purification, only α and β tubulin bands are seen on 5% SDS-polyacrylamide gels run in 25 mM Tris-glycine, pH 8.3. In 30–40% of the purified tubulin preparations, however, a single high molecular weight protein co-purifies with tubulin. This high molecular weight protein does not co-purify stoichiometrically with tubulin but varies from zero to a molar ratio of 1:40, and it co-migrates with ciliary and flagella dynein-1. The dogfish brain high molecular weight protein is not necessary for assembly, and its presence does not influence the assembly characteristics of tubulin. The critical free tubulin concentration necessary for self-assembly is approximately 0.2 mg/ml. Pelleted microtubules resuspended in cold 0.1 M MES buffer without Mg^{++} re-polymerize after clarification by centrifugation and re-warming, suggesting that only catalytic amounts of Mg^{++} are required for assembly. Mg^{++} in the range of 0.5 to 3.5 mM has no effect on the rate and extent of polymerization nor the critical concentration needed for assembly. After assembly is complete, a small fraction of the microtubules appear twisted as observed by negative staining, so that the protofilaments lie at a slight angle to the long axis of the tubule. In addition to the twist, the tubule has a periodic repeat of 96 nm axially. The repeating structure appears as a 48 nm wide spiral band which may arise from a helical perturbation of the basic microtubule lattice. The straight and twisted configurations may represent two functional states of cytoplasmic microtubules.

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In situ *experiments on* Spartina alterniflora *detritus decomposition and factors which control meiofaunal and microfloral abundance and community structure.* JOHN J. LEE, JOHN H. TIETJEN, CARMINE MASTROPAOLO AND MONICA LEE.

Four separate sets of experiments were incubated in greater Sippewissett salt marsh. One experiment studied the possible influence of macrofaunal grazing or predation on the assemblages of smaller organisms in the community. Cages to include or exclude macrofauna were 1 m^2 in area, 0.5 m above the sediment, and penetrated the sediment 0.2 m. The cages were constructed of plexiglass and had fiberglass-screened windows. They were placed in a 1 m tide pool so that sampling hatches, on the top of the cages, were just emergent at low tide. Caged were *Nassarius obsoletus* and *Fundulus heteroclitus.* Controls were cages which excluded grazing macrofauna, cages with plastic bottoms, or with no screens. Weekly samples were taken of the upper 3 cm of sediment inside and outside the cages. Estimates were made of the abundance of meiofauna, microphytes and microorganisms; the reproductive rates of the microorganisms and microflora; primary production of the microflora; diatom population structure; and heterotrophy among the microflora and microorganisms. Initial analysis of the data indicates that total meiofaunal population in cages with *F. heteroclitus* was only a small fraction (~25%) of that in controls. No significant differences were observed in primary productivity or heterotrophic uptake among the microflora or microorganisms in various cages. Other data will be analyzed in the coming year.

Smaller cages (100 cm^2) were used to study the effects of grazing pressure by *Bittium alternatum* on meiofaunal densities.

The effects of micro/meiofauna on the decomposition rates of *Spartina alterniflora* detritus were also studied. A mixture of natural and radionuclide labeled (^{14}C) *Spartina* detritus was incubated in an *in situ* plastic chemostat of our own design. Nylon filters (1.0 μm pore size) separated the contents of the vessel from the surrounding sea. Changes in microbial biomass, reproduction rates, C/N ratios and decomposition rates were estimated weekly. During decomposition of more refractory plant materials, the rate of decay was ~40–60% higher in vessels containing micro/meiofauna than those without them.

An experiment aimed at studying the stochastic features of natural benthic diatom assemblage trajectories was also incubated *in situ* in a marsh tide pool. We estimate that analysis of this data will take several years.

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Conformational equilibria in bacterial luciferase. ETHAN A. LERNER, MATTHEW P. FROSCH, MARGARET V. MERRITT AND THOMAS O. BALDWIN.

Bacterial luciferase from *B. harveyi* catalyzes the luminescent oxidation of $FMNH_2$ and a long-chain aldehyde. Luciferase is exceedingly susceptible to protease activities at low phosphate concentrations: the α-subunit is selectively degraded while the β-subunit is relatively stable under nondenaturing conditions.

Using refined micro-slab SDS-polyacrylamide gel electrophoresis we have found that the α-subunit is converted by trypsin into numerous transient species of molecular weight 33,000 to

25,000 daltons. Chymotrypsin appears to cleave at fewer sites yielding two fragments of approximately 30,000 daltons, one of which appears to be a digestion product of the other, and several smaller fragments at early times. During prolonged digestion these bands are further degraded, and in time the β-subunit is also degraded. When α-mutants AK-6, AK-9, AK-20, and AK-2H were analyzed, all gave altered fragmentation patterns relative to the wild type, but only AK-9 was greatly changed. Interestingly, the β-subunit of FB-1, a weak flavin binding β-mutant, is rapidly degraded by both proteases. This observation demonstrates quite clearly that the conformation of the β-subunit has been substantially altered by the lesion. When the wild type enzyme is digested to 1% of its original activity and excess FB-1 is added to this preparation, a gradual return to between 10 and 15% of the original wild type activity is recorded. This occurrence is most likely due to native α-subunit of FB-1 combining with undigested β-subunit of the wild type; an alternative explanation is that partially digested wild type β-subunit is still active. Sedimentation velocity data indicate that the digested enzyme maintains its overall physical integrity. We have utilized the ESR signal from a nitroxide spin-label introduced onto the α-subunit *via* a maleimide group to determine the relative polarity and solvent accessibility of the suspected aldehyde binding site. Furthermore, we are in the process of sequencing the α-subunit.

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Ultrastructure of the syncytial intestinal epithelium of a parasitic nematode. L. M. LEWIS, E. PANITZ, P. L. KRUPA AND R. SMITH.

The ultrastructure of the intestinal epithelium of *Strongylus edentatus* is similar in many respects to other nematodes. That is, it is a highly polarized structure that is divided into a number of regions: apical brush border of microvilli, subapical terminal web, mitochondrial region, and basal labyrinth and lamella. Silver proteinate staining reveals glycoprotein in the glycocalyx and within dense granules, as well as glycogen in most regions of the cytoplasm. An apparently unique structure is a paracrystalline body in the nucleus.

The syncytial epithelium has a greatly thickened terminal web (4 μm thick). In the related genus *Cyathostoma*, a similar terminal web was considered by another author to be an adaptation for mechanical support of the syncytial epithelium. Contractile movements of mammalian microvilli have been linked by other workers to an actin-myosin system in the microvillar and terminal web regions. Two models of contraction similar to striated muscle have been proposed. In one model, the zonula adherens are thought to function as attachment sites for actin microfilaments. In another model, actin microfilaments from adjacent microvilli are proposed to interact with each other without attachment to zonula adherens. Since the syncytial epithelium studied here lacks zonula adherens, any movements of its microvilli would support the second hypothesis. Further, since the intestine of nematodes lacks a muscle layer, the greatly thickened terminal web may serve to contract regions of the entire structure in addition to probably functioning in the movement of microvilli.

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Presynaptic calcium currents in squid stellate ganglion: a voltage clamp study using TTX and TEA. R. LLINAS, K. WALTON AND M. SUGIMORI.

In a previous voltage clamp study (Llinas *et al.*, 1976, *Proc. Nat. Acad. Sci. U.S.A.*, **73**: 2918–2922) of the magnitude and kinetics of the inward calcium current in the presynaptic terminal, tetrodotoxin (TTX) and 3 aminopyridine (3AP) were utilized to block sodium and potassium conductances, respectively. While 3AP has the advantage of blocking potassium when applied externally, its use is limited to small depolarizing voltage steps. This is because its blockage of G_K is incomplete at voltage levels above −10 mV. TEA produces a more complete blockage of G_K but must be injected intracellularly. To facilitate the latter, TEA was injected presynaptically using two electrodes such that the rather large iontophoretic current flowed between the electrodes rather than across the membrane. The presynaptic terminal was then clamped using two or three electrodes. From these experiments the following conclusions were reached: (a) the kinetics for the calcium currents at low depolarizations were the same as those obtained with 3AP; (b) $E_{Ca^{++}}$ was found to be 120–140 mV from resting at 10 mM $[Ca^{++}]_o$; (c) no outward calcium current was detected with Vm larger than $E_{Ca^{++}}$; (d) the time constant for the tail current ranged from 380 to 420 microseconds; (e) a study of the instantaneous currents indicated ohmic properties for the calcium channel; and (f) the relationship between the calcium current and the EPSP amplitude or rate of rise were both found to be close to linear with slopes between 1.2 and 1.5 using double log coordinates.

Studies on sperm-jelly interaction: is there chemotaxis? ALINA LOPO, CHARLES P. BEAN AND DAVID EPEL.

An involvement of chemotaxis in the "swarming" or isoagglutination response of sea urchin sperm to egg jelly has been proposed in the past but never demonstrated. The hypothesis that initial sperm-jelly interaction causes the release of a secondary attractant which in turn brings more sperm to the source was tested. The assay system consisted of a 5.5 cm falcon plastic dish with four wells, each containing a different test substance: sea water, sperm, solubilized egg jelly, or jelly and swarming sperm. The wells were covered with a 300 Å nuclepore filter on which a 1000 Å layer of gold had been evaporated. The gold causes all sperm that come in contact with it to become irreversibly attached. To this system 7 ml of a suspension of freshly spawned sperm was added (1×10^7 sperm/ml). After waiting a few minutes sperm attached to the gold surface over each well were counted. Sperm attached to the surface over the well containing swarming sperm in four times as great a frequency as over the other wells. In a second series of experiments we coated a gold-plated falcon plastic dish with monomolecular layers of egg jelly and ovalbumin, leaving a plain gold-plated area around each test protein. Following addition of a sperm suspension counts were made. The gold surface quickly became covered with sperm tightly bound to it. Initially, numerous sperm were seen over the jelly-coated area, although they were not bound to the surface. After a few minutes, the sperm dispersed from this area. No sperm were seen over the ovalbumin coated area at any time. However, visual observation of the jelly-gold and ovalbumin-gold interfaces revealed many more sperm bound to the gold immediately adjacent to the monomolecular jelly layer. We are interpreting the above observations as an indication of release of a diffusible sperm attractant following initial interaction of sperm with egg jelly. Although we have no data at this point on the nature of the signal, we have some evidence that jelly is degraded following exposure to sperm. More work is needed to determine if jelly breakdown products are the secondary attractant.

This study was performed in the Embryology Course at the Marine Biological Laboratory. Course was supported by NIH Training Grant HD-07098.

Osmotic regulation and Na+K-activated ATPase in the green crab, Carcinus maenas, *and the spider crab* Libinia emarginata. LINDA H. MANTEL AND JEFFREY LANDESMAN.

The portunid crab *Carcinus maenas* is a euryhaline hyperosmotic regulator. Osmotic concentration of hemolymph in this crab remains 100–300 mOsmol above that of the medium in salinities below 80% sea water. It is isosmotic in sea water. *Libinia emarginata,* a spider crab, is less tolerant of reduced salinity and must be acclimated slowly to dilute sea water. It is isosmotic to the medium at all salinities between 60 and 100% sea water.

To investigate possible differences in mechanisms of osmotic regulation between these two crabs, we measured Na + K-activated ATPase in gills and gut of the animals. In epithelia of crustaceans, this enzyme can use either K or NH_4 as a counterion for transport of Na. When crabs are acclimated to 100% sea water, specific activity of ATPase in gills of *C. maenas* is ten times as high as that in gills of *L. emarginata.* Posterior gills in *C. maenas* have 2–3 times as much enzymatic activity as do anterior gills, while in *L. emarginata,* activity in anterior and posterior gills is about equal. In both crabs, the foregut and midgut contain substantial activity of ATPase, with specific activity in gut of *L. emarginata* about the same as that in *C. maenas.* Possible role of the gut in osmoregulation in these crabs has not yet been assessed.

When crabs are acclimated to dilute salinity, specific activity increases in all tissues. Per cent activation of the enzyme is 140–150% in posterior gills of *C. maenas* and 125% in gills of *L. emarginata.* Specific activity in the gut increases by 130–150% in both crabs. Results in *C. maenas* are in contrast to those in *Callinectes sapidus,* where enzyme activity in the posterior gills increases by more than 200% on acclimation.

Although the level of ATPase in gills of the osmoconformer is far below that in the osmoregulator, both crabs are able to increase specific activity of the enzyme in dilute sea water. Thus, further study of both conformers and regulators is necessary to understand the relationship between osmoregulation and activity of Na + K-activated ATPase.

Supported by funds from City University of New York.

Evidence for synthesis of a polypeptide similar to human chorionic gonadotropin in aquatic organisms. TAKESHI MARUO, SHELDON J. SEGAL AND S. S. KOIDE.

The luteotropic glycoprotein produced by the placenta has been termed "human chorionic gonadotropin" (hCG) and has been considered to be a specific hormone of pregnancy, except for its production by various malignant tumors. A broader distribution of the active gene(s) for the synthesis of this molecule is indicated by recent reports of hCG-like material in cultures of Actinomyces, *Progenitor cryptocides* and in extracts of several nonplacental human tissues. Now, we report the presence in both vertebrate and invertebrate aquatic organisms of a nonglycolated polypeptide which displays hCG-like activity in radio-immunoassay (RIA) and radioreceptor assay (RRA), but not in the immature mouse uterine weight bio-assay (IMA). In visceral organs of the clawed toad, *Xenopus laevis* and the lady crab, *Ovalipes ocellatus,* the concentrations of hCG-like material are approximately 50% of those found in the term human placenta. Negative assays or only trace amounts of activity were found in total extracts and specific organ extracts of the dogfish (*Squalus acanthias*); polychaete (*Chaetopterus pergamentaceus*); sea urchin (*Arbacia punctulata*); starfish (*Asterias forbesi*); and sea cucumber (*Leptosynapta inhaerens*). The RIA was done with hCG, hCG-alpha, and hCG-beta. The RRA utilized bovine corpus luteum membranes.

First trimester placenta and term placenta contain 176 ng and 30 ng per mg of lyophilized extracts, respectively, when tested in RRA. A 50% displacement of ^{125}I-hCG in the RRA system is obtained with 1.5 ng of purified hCG. Based on this standard, crab stomach and hepatopancreas contain amounts of hCG-like substance equivalent to 15 ng and 10.7 ng per mg of lyophilized extracts, respectively. By RIA the first trimester placenta and term placenta contain 250 ng and 61.5 ng per mg of lyophilized extracts. A 50% displacement of ^{125}I-hCG in the RIA system is achieved with 0.8 ng of purified hCG. The amounts of hCG-like substance in crab stomach and hepatopancreas are estimated to be 14.2 ng and 13.3 ng per mg of lyophilized extract, respectively.

Physicochemical properties of the hCG-like substance in crab stomach extract were examined by affinity chromatography on a Con A-Sepharose column and gel filtration on Sephadex G-100. In contrast to chorionic gonadotropin in placenta extract, the hCG-like substance in crab stomach is not adsorbed by the Con-A-Sepharose column, suggesting that this substance contains little or no carbohydrate moieties.

The hCG-like substance found in the crab stomach has no biological activity when assayed by the mouse uterine weight method.

Direct effects of temperature on protein synthesis in vivo. RITA W. MATHEWS.

Studies of temperature dependency of protein synthesis have yielded widely varying results. In reticulocytes Craig has found Q_{10}'s of 2 and 3 for elongation rate and total protein synthesis respectively, at 24°–40° C, and Q_{10} about 8 for both processes at 10°–24° C. Complete cessation of protein synthesis is observed in *E. coli* at 8° C. The present study was carried out in the toadfish (*Opsanus tau*), a eurythermal marine species with a wide temperature tolerance (0°–30° C). Fish acclimated to ambient summer temperature (22° C) were transfered to the desired temperature 1 hr before experiment. Under MS-222 anesthesia, fish were injected with

^{14}C-leucine and ^{3}H-inulin into the hepatic portal vein. After incubation for 1 to 15 min, depending on temperature, livers were rapidly excised and fractionated to obtain total incorporation into protein and the distribution of label between completed proteins and ribosome-bound chains. The latter was used to determine polypeptide chain assembly time or elongation rate. In the temperature range 17°–30° C both elongation rate and total protein synthesis showed $Q_{10} = 2.5$. Extrapolation of elongation rate data by means of an Arrhenius plot to 37° C yielded six amino acid residues/sec, the value found in rat and mouse liver. At temperatures of 7°–17° C, Q_{10} for elongation increased to 5.0. Little incorporation occurred at lower temperatures (4°–7° C), and elongation rate could not be determined. The results indicate three types of protein synthetic function in toadfish: first, highly active system with moderate Q_{10} in the temperature range 17°–30° C—this system functions as well as that of mammalian liver; secondly, an impaired system showing an elevated Q_{10} in the range 7°–17° C; and thirdly, a nearly inactive system at temperatures below 7° C.

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Actin heterogeneity in the brush border of intestinal epithelial cells. PAUL T. MATSUDAIRA AND MARK S. MOOSEKER.

Using the technique of isoelectric focusing, actin heterogeneity was examined in the brush border of chicken intestinal epithelial cells. When prepared from acetone powders, the brush border actin separated into two major components present in a 1 : 1 ratio, and a third minor band. The major bands, comprising 95% of the total protein, co-migrated with the β and γ bands of smooth muscle actin from chicken gizzard, but not with the α actin band from chicken skeletal muscle. The same pattern was also obtained when whole brush borders, washed with Triton X-100, were isoelectric focused. (We have used the α, β, γ nomenclature of Whalen, *et al.*, 1976, *Proc. Nat. Acad. Sci. U.S.A.*, **72**: 2018–2022; and Rubenstein and Spudich, 1977, *Proc. Nat. Acad. Sci. U.S.A.*, **74**: 120–123.) In addition, the focused β and γ actins from the brush border comigrated with skeletal actin (α actin) when electrophoresed in the second dimension in the presence of SDS. The minor band, comprising 5% of the total protein in isoelectric focusing gels of brush border actin, occurs in the very acidic region of the gel. The identity of this minor band which was also seen with either smooth or skeletal actin is undetermined.

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Kinetic evidence for a common photosynthetic step in diverse seaweeds. DAVID MAUZERALL AND MICHAEL MISHKIND.

Evidence for the assumption that the mechanism of photosynthesis is the same irrespective of the kind of pigments in the photosynthetic unit was obtained by measuring the fast turnover time of O_2 production. The seaweeds were freshly collected from Great Harbor, Woods Hole. The turnover time was measured by the delayed double flash technique (Diner and Mauzerall, 1973, *Biochem. Biophys. Acta*, **305**: 353) using a stabilized O_2 electrode and a water bath (23° C). The single turnover saturating flashes also allowed measurement of the size of the classical Emerson-Arnold photosynthetic unit. The data are given as seaweed name, turnover time in msecs, and photosynthetic unit size (number of chlorophyll molecules per O_2 yielded per flash): *Ulva lactuca*, 0.43 ± 0.01, 1900; *Codium fragile*, 0.34, 1900; *Porphyra umbilicalis*, 0.52 ± 0.07, 600–1000; *Chondrus crispus*, 0.46, 1100; *Champia parula*, 0.44, 6000; and *Fucus vesiculosis*, 0.40, 1200. The errors are rms on those samples measured 21 and 6 times, respectively. The similarity of these turnover times to that of *Chlorella vulgaris*, 0.64 msecs, and *Phoridium luridum*, 0.54 msecs, is strong evidence for the similarity of the electron transport chain from photosystem II to the plastoquinone pool in all algae despite differences in pigmentation. The size of the photosynthetic unit is the same as that of *Chlorella* (2000) for the green seaweeds and about one half that of *Chlorella* for the red and brown seaweeds due to the extra antenna pigments (phycobilins and carotenoids). *Champia* is an exception, but only a single measurement was made. The turnover time did not change in sun and shade adapted *Ulva* and *Porphyra*.

This work was supported by the Experimental Marine Botany Class and NSF grant PCM74-11747.

Evidence that the diurnal cycle of photosynthetic capacity in Ulva *is caused by a dark reaction.* MICHAEL MISHKIND AND DAVID MAUZERALL.

Analysis of the light saturation curves and photosynthetic unit sizes for O_2 evolution shows that the diurnal cycle of photosynthetic capacity in *Ulva lactuca* occurs by changes in the rate of a dark reaction of photosynthesis. A highly sensitive O_2 electrode and water bath (23° C) allowed measurement of O_2 production at light intensities (0.2–2 mW/cm^2) such that the initial linear slope of the light saturation curve could be accurately determined. Since the average thallus absorption changes by less than 20% during the cycle (Mishkind, Mauzerall and Beale, 1976, *Biol. Bull.*, **151**: 420) this slope is equivalent to the relative quantum yield. A change in the rate of a light reaction directly alters the quantum yield. Samples were collected from Great Harbor, Woods Hole, for immediately assay. The relative quantum yields of 6 noon–midnight pairs were constant (± 5%) while their light saturated rates (per mole chlorophyll) cycled with an amplitude of 2.0. A pair of samples collected from the same frond had light saturated rates of 5 and 1.6 moles O_2 per mole chl per min at noon and midnight, respectively, but relative quantum yields differed by less than 20%. A change of 300% in the relative quantum yield would be expected if a light reaction was the limiting step in the diurnal cycle. In addition, the yield of oxygen from single turnover saturating flashes, that is, the size of the Emerson-Arnold photosynthetic unit, was constant (2360 ± 140 chl/O_2 for this sample) indicating that the size of the light-harvesting antenna is constant during the cycle. By analogy with *Chorella*, the limiting steps in photosynthetic electron transport involve the plastoquinone pool. Using the repetitive double flash technique (Diner and Mauzerall, 1973, *Biochem. Biophys. Acta*, **305**: 353) the fast turnover time for O_2 production was found to be constant during the cycle (0.48 ± 0.03 msec at 23° C). Thus the site of regulation is after the plastoquinone pool–system II step.

This work was supported by the Experimental Marine Botany Class and NSF grant PCM74-11747.

The nitrogen uptake kinetics and growth response of Spartina alterniflora. JAMES T. MORRIS.

The marsh grass *Spartina alterniflora* was grown hydroponically in continuously flowing nutrient solutions of constant composition. The solutions were enriched with nutrients in such a way as to insure that only nitrogen was growth limiting. The input concentration of nitrogen to three treatments was 714 μg-atom/liter, and the flow rates between treatments were varied to provide constant nitrogen supply rates ranging from 81.5 to 163.0 μg-atom per liter of culture solution per day. The constant supply of nutrients to hydroponic cultures is analogous to the turnover of nutrients in natural systems.

The supply rate of nitrogen had a significant effect on productivity, and production at the highest supply rate was greater than that found in local marshes. The temporal pattern of weight-specific growth rates of plants from each treatment showed a similar pattern, notably, rapid growth in the early summer followed by a rapid decline in growth rates that occurred later in the growing season, but earlier at progressively lower nitrogen supply rates. Weight-specific growth rates of all plants were initially equal indicating that growth was unlimited by nitrogen when the plants were small. The data suggests that the differences in plant yields arose primarily as a result of differences in the time at which the rapid decline in growth rates occurred.

There was no correlation between the temporal sequence of growth rates and either temperature or light patterns, but there was an excellent correlation between growth rates and absolute rates of nitrogen uptake. This suggests that the temporal pattern of growth rates was determined by rates of nitrogen uptake. The sudden decrease in growth rates appears to have corresponded to the time at which a transition occurred between high velocity and low velocity uptake mechanisms for nitrate, the principal nitrogen source. The concentration of nitrogen in the cultures decreased over time and resulted from the difference between supply and demand. The rate at which the concentration decreased was inversely proportional to the supply rate, which explains the temporal differences that existed between treatments with respect to decreases in growth rates.

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MODELSEA: towards a simulation model of particulate organic carbon flow in the open ocean. JOHN F. MURATORE AND RICHARD L. HAEDRICH.

Biological oceanographers have long been interested in the flow of materials through marine ecosystems. The release of toxic substances into the oceans has made this a topic of great applied importance. MODELSEA is an attempt to use a deterministic specific process numerical simulation model to consider the process of particulate organic carbon (POC) flow in the open ocean. MODELSEA considers the flow of POC in a 2000 m deep column of water under a hectare of open ocean. In the model, detritus is generated at various depth levels as a function of the standing crops of various organisms, is eaten by organisms, decays and sinks. Detritus is generated for a single day and is then tracked until it either hits bottom or is consumed. The sum of material reaching the bottom over the simulation equals the daily input to the bottom without making assumptions about the distribution of detritus in the water column. The food web used in the model is nontrivial and is intended to represent a generalized open ocean food web.

When the model is run with best estimates from the literature for all parameters, it predicts a flow of 1.45 g material/($m^2 \cdot$day). The dominant component is zooplankton fecal pellets [1.40 g/($m^2 \cdot$day)]. This compares to measured rates in the literature of between 74 and 242 mg material/($m^2 \cdot$day). The difference between model predictions and observations may be due to both sampling problems and underestimates of feeding on fecal pellets in the model. Model predictions also suggest that approximately 1 gram of dead fish carcass should be reaching each 25 m^2 of bottom per day. This suggests two possible life styles for deep benthic organisms. The first is keyed to small and more abundant fecal pellets, and the second keyed to larger fish carcasses.

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Formation of gap and tight junctions between reaggregated blastomeres of Fundulus, *a freeze fracture study.* Z. NE'EMAN, M. E. SPIRA AND M. V. L. BENNETT.

Cells from late blastulae were dissociated in 2X Holfreter's solution with 0.1% colchicine by several passages through a Pasteur pipette and gently centrifuged to form a pellet. After different intervals pellets were fixed overnight in a glutaraldehyde, paraformaldehyde and acrolein mixture and processed for freeze fracture. Intact blastulae in the same solution show clusters of gap junctions of variable size (0.1–2 μ diameter). Apical tight junctions in multistranded zonules are found only between superficial cells. These zonules separate lateral membrane with ordinary numbers of particles from apical membrane which is particle free and of relatively high electrical resistance and low water permeability. Five to eight min after reaggregation no gap or tight junctions are seen; particle aggregates of intact blastulae have disappeared. After 10–15 min, isolated small gap junctions (0.1–0.2 μ diameter) occur. After 26–60 min, gap junctions are somewhat larger (0.15–0.35 μ diameter) but still not in clusters. Formation apparently proceeds by adding particles; we did not see specialized formation plaques involving larger particles as reported by others (Decker and Friend, 1974, *J. Cell Biol.*, **62**: 32–47; Johnson *et al.*, 1974, *Proc. Nat. Acad. Sci. U.S.A.*, **71**: 4536–4540). The time course is consistent with electrophysiological measurements of coupling between reaggregated blastomeres of earlier stages. Isolated tight junction strands appear after 15 min. There are also complex whorls and elaborate networks of strands some of which are larger than in intact embryos.

The incidence is higher in aggregates, which suggests that some cells are forming tight junctions that would not do so *in situ*. All images are consistent with growth of strands by adding particles at loose ends. Where zonular tight junctions occur, membrane on one side is free of particles as in intact embryos. Sometimes particle free and ordinary membrane are not separated by a tight junction although occurring near one. This system provides new information as to formation of junctions and should prove useful for further experimental studies.

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Effects of thyroid hormone on elongation factor 1 levels in toadfish liver. JENNIFER B. K. NIELSEN.

Thyroid hormone stimulates protein synthesis in vertebrates and is implicated in the ability of homeotherms to withstand acute cold. This project is concerned with the possible involvement of thyroid hormones in the process of cold acclimation in a teleost fish. Cold-acclimated toadfish show increased liver protein synthetic rate relative to warm-acclimated fish when both are measured at the same temperature. The increased rate of polypeptide chain elongation has been correlated with tissue concentrations of elongation factor 1 (EF-1). No change was observed in ribosome content or in elongation factor 2 (EF-2) levels. The rise in EF-1 activity within 10–14 days after transfer of toadfish to low temperatures occurs primarily in the lower molecular weight forms of EF-1 (50,000–150,000 daltons).

In the present study EF-1 in liver post-mitochondrial supernatants was assayed in a polymerization system containing salt-washed rat liver ribosomes, ^{3}H-phenylalanyl tRNA, poly(U), and an excess of highly purified EF-2 from *Artemia salina*. Although toadfish ribosomes are present in post-mitochondrial supernatant, they did not have a significant effect on the assay compared with assays of ribosome-free supernatants. The injection of 5 μg triiodothyronine (T_3)/100 g fish every other day for two weeks caused a raise in specific activity of EF-1 from a control value of 7.0 ± 1.8 ($N = 25$) pmoles/(min·mg protein) to 10.4 ± 2.0 ($N = 5$). The distribution of large (400,000–700,000 daltons) and small forms of EF-1 changed from 50/50 in the control to 30/70 in T_3-treated fish. Thus, the increased activity was associated with small forms, as in cold acclimation. The rise in activity occurred at about 12 days. A dose of 10 μg/100 g fish caused a slight elevation in the EF-1 level to 9.3 ± 0.3 ($N = 3$), large/small = 42/58. Doses of 20 and 40 μg/100g fish gave activity levels and molecular weight distributions identical to untreated controls.

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Protein release from the internal surface of the squid giant axon membrane during excitation and during application of agents which modify excitation. HARISH PANT, SUSUMU TERAKAWA, JESSE BAUMGOLD AND ICHIJI TASAKI.

The protein in the perfusate collected from intracellularly perfused squid giant axons was analyzed after being labeled with radioactive ^{125}I-Bolton-Hunter reagent. The amount of released protein increased by a variety of manipulations which modify excitability: first, depolarization of the axon with high concentration of externally applied potassium ions; secondly, repetitive stimluation of the axon perfused with either TEA containing solution or with normal KF containing solution; thirdly, replacement of potassium ions in the perfusion solution with sodium, caesium or tetraethyl ammonium (TEA); and fourthly, perfusion with alkaline (pH above 9.0) solution. In the case of potassium depolarization, the molecular weight distribution of the proteins analyzed by SDS polyacrylamide gel electrophoresis showed major peaks around 100,000; 60,000; 43,000 and 12,000 daltons. The other cases yielded patterns in which the 43,000 and 12,000 dalton peaks were dominant. The released proteins were not believed to derive from the remaining axoplasm, since SDS polyacrylamide gel electrophoresis of isolated axoplasm proteins gave different molecular weight profiles with at least 18 major bands of which the following were dominant: 200,000; 170,000; 40,000–59,000; and 12,000–35,000 daltons. These observations indicate that there exists a group of proteins closely associated with the axonal membrane which can be released by a variety of factors which modify membrane excitability.

Evidence that acetylcholine in subfractions of squid brain synaptosomes is contained in an osmotically sensitive compartment. HARVEY B. POLLARD, ROBERT B. HANNA AND GEORGE D. PAPPAS.

Synaptic vesicles are assumed to be osmotically inactive structures and are prepared from synaptosomes by hypotonic lysis in distilled water. During this process, nearly 80% of the endogenous Ach and most of the newly synthesized Ach is rendered soluble, and it has been concluded by a number of investigators that much of the Ach in resting and stimulated nerve terminals is cytoplasmic. However, this conclusion would be untenable if vesicles were, in fact, osmotically sensitive.

To approach this problem, we tried to prepare broken synaptosome preparations that had Ach in a particulate form. A glycerol-lysis technique, developed for preparing serotonergic granules from human platelets, was modified for use with squid brain synaptosomes. The technique involved equilibration of synaptosomes with a solution that was 4 M glycerol/M glucose, followed by transfer to a solution containing M glucose only. The initial homogenization step for preparation of synaptosomes resulted in a 50% loss of total Ach, but most of the remainder was within synaptosomes. Exposure of the synaptosomes to the 4 M glycerol/M glucose solution resulted in no loss of Ach. However, omission of the glucose from the mixture, leaving only the permeant glycerol, resulted in complete loss of all detectable Ach. This alone was good evidence for an osmotically sensitive compartment of Ach.

Glycerol-loaded, intact synaptosomes were then rapidly diluted into M glucose, with no detectable loss of Ach, and the Ach activity was then found to be capable of sedimenting through a M sucrose step at 100,000 × *g* for one hour. It is concluded from these studies that Ach in squid brain is in an osmotically sensitive compartment that is recoverable from a broken synaptosome preparation. Subsequent purification steps and ultrastructural analysis will reveal if this compartment is identical to synaptic vesicles.

An ecological model to determine the factors controlling decomposition: a bioenergetic approach. JAMES P. REED.

A model of decomposition has been developed during the early stages of an active field sampling program designed to determine the factors controlling decomposition in a salt marsh. The development of the model has necessitated quantification of all the components that are believed to be important. In doing so, this brought out the strengths and weaknesses of the data base and pointed to the areas where further research is required. A significant portion of the model is devoted to evaluating such seasonal functions as light, temperature, wind speed, salinity and tides and their effect on factors such as current speed, particle suspension, evaporation, percolation, etc. The biotic portion, as influenced by the latter, couples the actions of algae, macrophytes, and macro-invertebrates with the microbes. The microbial section serves as a vehicle to oxidize or reduce compounds and to produce or reduce the bacterial populations. These processes are controlled by the bioenergetic and nutritional potentials of the system. Based on Gibbs free energies, nitrogen sources, the cell decay and turnover rates, the stoichiometry of the reaction from electron donor to acceptor (plus new cells) is derived. The uptake rate of each of the reactants is determined by the Monod equation. These rates divided by their stoichiometric coefficients determine the rate at which the reaction would go to completion if all the other reactants were in excess. The lowest of these latter rates is then taken to be the actual rate at which the reaction goes to completion. The model integrates these rates, determines the new concentration of materials, increments time, and continues to run through the system of equations until the finish time. By manipulation of selected parameters in the model, their effect on decomposition can be assessed.

On the fate of synaptic vesicle membrane in photoreceptor terminals of the skate retina. H. RIPPS, M. SHAKIB AND E. D. MACDONALD.

It is now widely accepted that exocytosis is the mechanism by which synaptic vesicles, as well as other secretory granules, discharge their soluble contents into the extracellular space.

Since the vesicle membrane is necessarily incorporated into the plasma membrane, the cell must have a means of regulating its total surface area, as well as maintaining a healthy supply of transmitter-charged synaptic vesicles for sustained action. Clearly, an efficient regulatory mechanism is one that provides for internalization and re-use of vesicle membrane following exocytosis. We have attempted to identify the steps in this conservation process by examining the ultrastructural changes in the receptor terminals of the skate retina that follow prolonged incubation in Ringer solutions containing 100 mM potassium propionate; the high concentration of K^+ depolarizes the photoreceptor and promotes the discharge of synaptic vesicles.

After 2 min in the high K^+ solution, there was only a small reduction in the number of synaptic vesticles, and no apparent change in the gross morphology of the terminal. There was, however, a remarkable number of vesicles that had fused with the plasma membrane and appeared to be in various stages of exocytosis. However, after 30 min in the high K^+ solution, profound morphological changes had occurred. The synaptic vesicle content of the terminal was reduced to about 40 per cent of its normal value, the surface area of the terminal had increased greatly, and the telodendria, which had expanded and had become tortuous, came to insinuate itself around glial cells and invade the body of the synaptic terminal. Thus, the membrane of expended vesicles had gone to form an extended axolemma.

It should be noted, however, that there was no significant increase in the number of cisternae, vacuoles, coated vesicles, or other cytoplasmic elements that have been implicated in the vesicle-recycling process. Moreover, no changes were detected in these constituents after the terminals were perfused with a normal Ringer solution that restored fully the former complement of synaptic vesicles. It appears, therefore, that a rapid exchange of membrane between axoplasmic vesicles and the plasma membrane provides the principal mechanism for conserving vesicle membrane at the photoreceptor terminal.

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Radioactive sodium efflux from internally perfused squid axons. V. SCRUGGS AND D. LANDOWNE.

We have investigated sodium efflux from giant axons of the squid *Loligo pealii* in the absence and presence of internally applied 50 micromolar veratridine under conditions in which the external sodium was replaced with lithium, Tris or choline. The axons were continuously perfused with a medium containing 275 mM KF, 70 mM NaF, 1 mM (K)EDTA, 30 mM (Na) HEPES and 255 mM mannitol to which a trace amount of ^{22}Na had been added. The artificial sea water flowing past the axon externally contained 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$, 5mM (Na) HEPES and 460 mM NaCl. The sodium substitutes replaced the Na on an equimolar basis. The axons were voltage clamped to a membrane potential of −60 mV.

When axons not exposed to veratridine were stimulated at 5/sec with 3–6 msec depolarizing pulses there was a large increase in sodium efflux compared to resting values. This extra efflux was decreased approximately 50 per cent when the external sodium was replaced with either lithium or Tris. However when choline was used as a sodium substitute there was no change in sodium efflux. This suggests that the reduced efflux is probably not the result of a simple sodium-sodium exchange mechanism unless the membrane process is unable to distinguish between choline and sodium.

When axons were exposed to 50 micromolar veratridine (a veratrum alkaloid known to induce a TTX sensitive sodium permeability) at a constant membrane potential of −60 mV the holding current became more inward, and there was a concomitant increase in sodium efflux. When the external sodium was replaced with lithium, tris or choline this extra sodium efflux was abolished.

Methods for study of protein metabolism in the rainbow trout. MICHAEL A. K. SMITH AND AUDREY E. V. HASCHEMEYER.

Rainbow trout, *Salmo gairdneri*, 300–400 g body weight, were obtained from the State Fish Hatchery (Sandwich, Massachusetts) and were acclimated to sea water for three weeks. Fish given standard trout feed *ad libitum* by means of a self feeder system showed growth rate 0.54 ± 0.2% body weight per day. Starved fish lost weight at the rate of 0.6 ± 0.2% per day. Analysis of protein synthetic rate in liver *in vivo* was carried out by three methods: first, determination of the fractional rate of incorporation of ^{14}C-leucine at times of 1 to 5 min after hepatic portal vein injection; secondly, determination of polypeptide chain elongation rate and ribosome concentration of liver; and thirdly, incorporation of ^{14}C-tyrosine in liver, gill and muscle following constant infusion of isotope for 6 hr in free-swimming fish. Experiments involving hepatic portal vein injection were carried out at 15° C in fish anesthetized with benzocaine. Constant infusion was done in fish cannulated in the dorsal aorta for infusion and in the caudal vein for blood sampling. No difference in incorporation results was obtained in fish used 2 hr or up to 5 days after cannulation. Cannulated fish showed normal swimming and feeding behavior. Results obtained to date in liver indicate that protein synthetic rates in fed trout are comparable, after correction for experimental temperature, to those observed in liver of toadfish and rat. Average polypeptide chain assembly time at 15° was 6.3 ± 1.0 min (15), corresponding to chain elongation rate of 1.1 amino acid residues/sec. Preliminary results obtained by constant infusion indicate a depression of liver protein synthesis (as per cent liver protein replaced per day) in starved fish but no change in gill protein synthesis. Assay of muscle data is in progress.

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Effect of sampling time on primary production estimates by the ^{14}C method. PATRICIA D. SMITH AND CLAUDE W. DEPAMPHILIS.

The ^{14}C method of estimating primary production is a sensitive technique and in wide use today. However, there exist unanswered some basic questions concerning what one is actually measuring. These include whether the estimate is of net or gross primary production and whether subtraction of the dark bottle uptake from that of the light is a proper correction for uptake due to nonphotosynthetic carbon-fixation. Unfortunately, these basic problems are coupled with technique problems.

Several studies have been done improving the efficiency of the technique at various steps, such as filtering small quantities of sample to minimize error due to cell rupture and exposing filters to HCl fumes to remove labeled bicarbonate ion adsorbed to the membrane. This study was designed to determine whether the time of sampling has a significant effect on the primary production estimate.

To test the variable sampling time effect, water samples were taken at sunrise, noon, sunset and midnight. At the time of sampling, the water was poured into light and dark bottles and inoculated with ^{14}C-labeled sodium bicarbonate. They were then incubated for 24 hours under conditions of 15 hours light and 9 hours dark.

The results show a significant difference at the 0.01 level in carbon-uptake rate with sampling time in the first run done on a sunny day, whereas no significant difference in carbon uptake rate was observed with sampling time in the second run done on a cloudy day. The difference in result between runs is possibly due to the low light intensity on the second day causing minimal phytoplankton migration.

These preliminary results indicate that the time of sampling may indeed effect the primary production estimate. This would imply the need to standardize the sampling time in the ^{14}C method. This, along with the other revisions, must be incorporated in the technique if the estimates by independent researchers are to be comparable.

Effects of temperature on the pupillary response of the butterfly eye. MANDYAM V. SRINIVASAN, GARY D. BERNARD AND DOEKELE G. STAVENGA.

Pupillary responses were evoked by monochromatic green flashes of light, and recorded by monitoring eyeshine with a deep-red, subthreshold light. At eye temperatures above the ambient 24° C, sensitivity of the pupil is reversibly reduced. A temperature increase of 10° C shifts the intensity-response function of *Nymphalis antiopa* by +0.3 log units, and that of *Eurema nicippe* by +0.5 log units.

Increasing the temperature speeds up closing and opening of the pupil. For example, in *Nymphalis*, a temperature increase of 10° C decreases the half-time of closing by 25%, and that of opening by 15%. In general, closing-speed is more temperature-sensitive than opening-speed. This feature is very pronounced in pierids. For example, in *Eurema*, a temperature increase of 10° C decreases the closing half-time by 55%, but there is no measurable change in the opening half-time. Similar results were obtained in *Pieris rapae* and *Colias eurydice*. This differential effect of temperature suggests that closing and opening are mediated by different mechanisms.

It has been hypothesized in the literature that the light-attenuating granules which mediate the pupillary response are in continuous, random motion within the retinular cells, and that this brownian motion mediates opening of the pupil in the dark. We have observed small, random fluctuations in the intensity of eyeshine, which have a power spectrum restricted to the range 0–2 Hz. This observation supports the existence of brownian motion. However, the importance of brownian motion as a mechanism for pupil opening is questionable. The finding that opening-speed is independent of temperature in pierids suggests that other mechanisms must be involved.

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Optics of compound eyes and circadian pigment movements studied by pseudopupil observations in vivo. DOEKELE G. STAVENGA.

Important structural and functional qualities of compound eyes can be inferred by studying pseudopupil phenomena. The (principal) pseudopupil of apposition eyes marks the ommatidia that are aligned with the direction of observation. When illumination and observation are not aligned, the oblique illumination pseudopupil, hitherto undescribed and unexploited, marks the direction of illumination. Principal pseudopupil and corneal reflection do not coincide when ommatidial axes are skew to the eye surface, if viewed with epi-illumination. It can be shown, *e.g.*, in damselflies, that skew ommatidia are utilized effectively to construct foveas and to achieve broader visual fields. Circadian movements of distal pigment are easily observed by examining the pseudopupil, *e.g.*, in crab, praying mantis, katydid, and *Limulus*. These pigment movements cause the ommatidial aperture to be enlarged at night. The day-adapted state can be established at night by either illumination or cooling.

The usually black color of the principal pseudopupil is due to the primary pigment cells, which absorb over a wide spectral range, and so act as an optical screen for the photoreceptors. The secondary pigment determines the eye coloring and, when this is different from black, hides the primary pigment from the attentive predator and/or prey. The red primary and secondary pigments of many red-eyed flies provide a means of photoregenerating the visual pigment. Still, the flower fly *Lathyrophthalmus aeneus* seems to follow the general characteristic that the eye's coloring is part of the animal's display features; the head resembles that of a heavily pollinated bee due to a yellowish coating over the red eye pigments.

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Primary structural differences distinguish cytoplasmic and central pair from outer doublet tubulins. R. E. STEPHENS.

Previous studies of the α and β subunits of sea urchin flagella outer doublet tubulins, utilizing comparative amino acid composition and peptide mapping, showed that the more strongly-associated tubulin of the A-tubule contained 2–3 more (lys + arg) residues in each chain than did the homologous subunits of the B-subfiber, with a correspondingly higher amount of certain small, hydrophilic, cathodic peptides being found in tryptic digests of A-tubulin subunits. This work has been extended to the tubulin of sea urchin sperm flagella central pair, derived by limited, low ionic strength dialysis of the 9 + 2 axonemes, and to cytoplasmic, vinblastine-precipitated tubulin from unfertilized eggs of the sea urchin *Strongylocentrotus* *droebachiensis.* The respective α and β chains were isolated by preparative SDS-PAGE and subjected to amino acid analysis and peptide mapping. The (lys + arg) content was higher in the central pair α chain than in the cytoplasmic counterpart, whereas the β chain had a lower content. Both chains from either source were significantly lower in these basic amino acids than their outer double counterparts. These differences were reflected in several unique peptides which distinguished both chains of cytoplasmic tubulin from those of central pair. The distribution of small, cathodic peptides was identical for the cytoplasmic and central pair subunits but differed markedly from that of the outer doublet tubulins. A very basic peptide, common to the outer doublet α and β chains, was absent while a less basic peptide was found only in cytoplasmic and central pair α chains. Thus cytoplasmic and central pair tubulin chains are structurally distinct from one another and from those of the outer fibers. These differences also allow one to conclude that no significant portion of the vinblastine-precipitable tubulin in the unfertilized egg is destined for cilia

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Tetraphyllidean and tetrarhynchidean metacestodes from squid, Loligo pealei *Lesueur, 1821.* HORACE W. STUNKARD.

Tetraphyllidean and tetrarhynchidean cestodes mature only in the spiral valve of selachian fishes. Both adult and larval stages have been known for more than one-hundred and fifty years. All have three-host life-cycles; developmental stages can be recognized, but no complete life-cycle has ever been experimentally demonstrated. Eggs of tetraphyllidean species do not hatch until they are eaten; those of tetrarhynchidean species are operculate, and swimming, ciliate, coracidia emerge. The first hosts are copepods. When the hexacanth oncosphere, either in an egg or in a coracidium, is eaten, it bores out of the intestine and transforms into a procercoid in the body cavity of the copepod. If the copepod is eaten, the procercoid bores out of the intestine and, in either the body cavity or adjacent tissues, becomes a plerocercoid. If the plerocercoid in its host is eaten, the worm, now a metacestode, bores through the intestinal wall and may encyst. It may pass from one host to another as successive hosts, invertebrates or fishes, are ingested, until it is eaten by a selachian, which is the final link in a food chain. The metacestode becomes the scolex of the definitive cestode and in several families the form and structural details are sufficiently developed to permit provisional identification.

Metacestodes have been taken from squid and eight species are recognized. Four are tetraphyllidean: slightly developed specimens are assigned to *Scolex pleuronectis* O. F. Muller, 1788; others are identified as *Phyllobothrium loliginis* (Leidy, 1887) Linton, 1897; *Dinobothrium septaria* van Beneden, 1889; and *Ceratobothrium xanthocephalum* Monticelli, 1892. Four tetrarhynchidean species are referred to *Nybelinia bisulcata* (Linton, 1889) Dollfus, 1929; *Nybelinia yamagutii* Dollfus, 1960; *Lacistorhynchus tenue* (van Beneden, 1858) Pintner, 1913; and *Otobothrium crenacolle,* Linton, 1890.

Investigation suuported by NSF BMS-74-14534.

Studies on messenger RNA template activity during early development of Lytechinus pictus. QUENTIN TONELLI AND TIM HUNT.

Sea urchin eggs make very little protein, and after fertilization there is a 10–20 fold increase in the rate of protein synthesis. Of the several possible mechanisms which might account for the relatively low activity of egg ribosomes compared with early embryos, we sought to examine the template activity of the mRNA known to be stored in mature eggs of *Lytechinus pictus* and to compare it with the mRNA from embryos at various stages of development.

RNA was prepared from eggs or embryos by hot phenol-SDS extraction at pH 8.8, precipitated by and washed with 75% ethanol, taken up in water and lyophilized. RNA preparations were tested for template activity in the mRNA-dependent reticulocyte lysate system, and translation products analyzed on SDS-polyacrylamide gels. No significant difference was detected in the template activity between the RNA extracted from eggs and embryos, and the products of translation closely resembled each other, and the proteins synthesized by a cell-free extract of *Lytechinus* 4-cell stage embryos.

This result indicates that modification of mRNA after fertilization is not the mechanism responsible for the activation of protein synthesis in this system.

This work was supported by NIH Training Grant HD 07098 and NIH Research Grant HD-06574.

Studies of the predominant nonfibrillar protein from scallop muscle: purification and properties. D. R. van der Westhuyzen, A. Stracher and D. A. Fischman.

Proteins of the striated adductor muscle of the scallop, *Aequipectin irradians*, were characterized by gradient SDS-polyacrylamide gel electrophoresis. The proteins soluble in a low salt buffer (0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Tris-acetate, pH 7.2) included one major polypeptide band, and several less abundant bands. The major polypeptide did not copurify with myofibrils, and was extractable from muscle with a minimum of homogenization and under conditions where myofibrils retained a good morphological appearance, including retention of the Z-band structure. This polypeptide had an estimated molecular weight of 48,000 daltons and was present in muscle at a concentration approximately equal to tropomyosin (5% of myofibrillar protein). The 48,000 dalton polypeptide was not present in significant amounts in the smooth adductor muscle of *Aequipectin*, the red adductor of *Mercenaria mercenaria*, or the leg muscle of *Limulus polyphemus*. It was easily purified from the soluble protein fraction to greater than 90% purity by precipitation between 60 and 85% ammonium sulfate saturation. Such a purified preparation was soluble at pH 8.0 or above. Purified protein had a low solubility at pH 7.0 even in the presence of high salt (1 M KCl), 1% Triton X-100, reducing agent or EGTA. At calcium concentrations of 1–2 mM or greater the protein was precipitated, and its electrophoretic mobility in SDS was accelerated slightly. Disappearance of 48,000 dalton protein, presumably due to proteolysis, occurred on incubation of whole muscle homogenate or the soluble fraction. Disappearance was dependent on the presence of calcium (1 mM) and was optimal between pH 8.0 and 9.0. Evidence, based on criteria of solubility, molecular weight and abundance, suggests that the protein does not correspond to a membrane-bound protein, calsequestrin, tubulin, 100 Å filament desmin-like protein, 55,000 dalton actin-binding protein, or any known myofibrillar protein. The function of the protein is unknown.

D. R. v.d. W. is a USPHS International Postdoctoral Fellow.

Isolation of an inhibitor of sponge aggregation factor. Jeffery W. Walker and Tom Humphreys.

An aggregation inhibitor has been isolated from *Microciona prolifera* following the procedures of Müller *et al* (1976, *J. Cell Sci.*, **21**: 227–241). Sponge tissue was extracted in 2 volumes of hypotonic saline containing 0.1% sodium dodecyl sulfate (SDS). After dialysis against 8 M urea to remove SDS and then against calcium and magnesium free sea water to remove urea, the extract was tested for its ability to inhibit aggregation caused by aggregation factor (AF). One unit/ml of inhibitor was defined as the concentration required to inhibit the promotion of aggregation caused by 4 units/ml of AF after several hours of aggregation at 22° C. Lower concentrations of inhibitor delayed the expression of AF activity. At greater concentrations the inhibitor also stopped normal aggregation of the cells which occurs at 22° C. The inhibitor did not prevent gel formation by the factor in the presence of 0.02 M $CaCl_2$. The inhibitor activity was nondialysable, insensitive to 100° C for 2 minutes, stable to digestion by 50 μg/ml of Proteinase K for 72 hours at 37° C, and soluble in 5% tricloracetic acid. Chromatography on Sephadex G-75 indicated a molecular size of several thousand daltons (dextran standard) and gave a five-fold purification of the partially included inhibitor. Five per cent TCA precipitated two-thirds of the protein and one-half of the polysaccharide from crude extract resulting in a 2.7 fold purification. The inhibitor was bound to DEAE-cellulose in 0.01 M NaCl and eluted with 0.5 N NaCl. A salt gradient covering this range showed activity eluting early and in one peak. Neither the component of the sponge yielding this inhibitor nor the site of its action on cells or factor are known at this time. This inhibitor will provide a new tool for the analysis of the sites on cells and factor which function during aggregation.

Supported by NSF Grant PCM-76-09309

Attack of Arbacia *eggs by dogfish phagocytes: a model of immune injury.* G. WEISSMANN, M. FINKELSTEIN, R. QUINN, L. TECHNER AND P. DUNHAM.

Phagocyte cells secrete lysosomal enzymes upon exposure to solid surfaces coated with immune complexes or aggregated immunoglobulins. Although this model has been related indirectly to antibody-mediated cytotoxicity, direct proof has been lacking. We used eggs of *Arbacia punctulata* as targets for cytotoxicity mediated by blood phagocytes from the dogfish *Mustelus canis.* Washed eggs in elasmobranch Ringer's solution were coated (30 min, 23° C) with dogfish IgM which had been chromatographically purified and heat-aggregated (15 min, 62° C). After mixing of coated eggs with phagocytes, cytolysis of the eggs was measured by the appearance in the suspending medium of cytoplasmic enzymes (catalase, superoxide dismutase, and β-glucuronidase), which were released in a time-dependent fashion for up to five hours. With Nomarski optics, attack of the eggs was observed: phagocytes adhered to coated cells, degranulated, and caused elevation of fertilization membranes in up to 40 per cent of the eggs. Lysis was then observed as damage radiating from the point of phagocyte-egg contact. By four hours, coated eggs exposed to phagocytes released 10.2, 11.9, and 7.5 per cent ($n = 9$) of total catalase, β-glucuronidase, and superoxide dismutase, respectively. This enzyme release exceeded by approximately two-fold that from uncoated eggs with phagocytes or eggs alone (uncoated or coated). Since activated eggs release a protease sensitive to soy bean trypsin inhibitor (SBTI), it was considered possible that this enzyme might be responsible for autolysis of the eggs. This possibility was excluded since first, lysis of eggs was not inhibited by SBTI, and secondly, the major protease of phagocytes, a trypsin-like enzyme (assayed using synthetic peptide substrates), was also not inhibited by SBTI. The results suggest that Ig-coated cells are killed when exposed to lysosome-rich phagocytes and that enzymes from attacking phagocytes, but not from the target cells, are responsible for cell death in this model of immune injury.

Studies of the change in NAD kinase activity during the activation of eggs of Arbacia punctulata. H. STEVEN WILEY, RANDAL N. JOHNSTON, DAVID BEACH AND DAVID EPEL.

The increase in NAD kinase activity after fertilization of *Arbacia* eggs is induced by agents which also increase the availability of intracellular calcium, but not by NH_4Cl, which causes an increase in intracellular pH. Using both measurements of the fluorescence of eggs *in vivo* and enzymatic assays of NAD and NADP in egg homogenates, NADP increases from 36 to 63 μM within 5 min of insemination of eggs, whereas NAD decreases from 210 to 180 μM. Treatment of eggs with 10 μM calcium ionophore A23187 induces similar changes in NAD and NADP, whereas eggs treated with 10 mM NH_4Cl exhibit no change in concentration of pyridine nucleotide. When eggs in 10 mM NH_4Cl are later treated with sperm or ionophore, the fluorescence increases to the same level as in controls. Thus, the increase in NAD kinase activity is associated with the increase in intracellular calcium, but not with the increase in intracellular pH. NAD kinase 500-fold was purified from unfertilized eggs using calcium phosphate chromatography, and no effect of Ca, Mg, EGTA, or EDTA was found on the enzymatic activity. Thus, the effect of Ca on the activity of the enzyme *in vivo* is probably indirect. The localization of NAD, NADP and NAD kinase was also examined after fractionation of homogenates of unfertilized and fertilized eggs on sucrose gradients. The results indicate that NAD kinase is present free in the cytoplasm of unfertilized and fertilized eggs. Consistent with this, mitochondrial NAD and NADP do not change after fertilization, whereas cytoplasmic NAD and NADP undergo a parallel decrease and increase, respectively, after fertilization.

This work was supported by NIH training grant HD-07098.

Sucrose-mediated uncoupling in Chironomus *salivary gland delayed by Ca^{++}.* EDWARD H. WILLIAMS AND BIRGIT ROSE.

Hypertonic sucrose has been previously shown to cause electrical uncoupling between cells and the splitting of gap junctions which are generally considered to be the structural basis for the low-resistance pathways mediating electrical coupling. The effects of hypertonic sucrose on electrical coupling in *Chironomus* salivary gland cells and the possible role of external Ca^{++} in the maintenance of the integrity of low-resistance junctions between cells in the presence of hypertonic sucrose were examined. The presence of Ca^{++} (12 mM) in the external medium protects *Chironomus* salivary gland cells from rapid electrical uncoupling by 0.5 M sucrose. In this condition, that is, the presence of external Ca^{++} and hypertonic sucrose, cells exhibit a depolarization of 8–25 mV and an increase in input resistance of 3–4 fold but no change in electrical coupling within 5–8 min. When Ca^{++} is replaced by Na^{+} and 1 mM EGTA is added, cells undergo complete uncoupling within 3 min with a 2–4 fold rise in input resistance and a 10–15 mV depolarization. However, in the absence of hypertonic sucrose, 1 mM EGTA and Na^{+} substitution for Ca^{++} caused a 10–15 mV depolarization but no change in electrical coupling or input resistance within 5–8 min. Several possible roles of external Ca^{++} in the prevention of immediate uncoupling by hypertonic sucrose may be considered. First, the absence of Ca^{++} might alter the intercellular adhesion properties between salivary gland cells so as to facilitate a physical separation of the cells when exposed to a hypertonic sucrose solution. Secondly, external Ca^{++} may be essential to stabilizing the linkage of the junctional elements themselves, protecting them from the action of hypertonic sucrose. Thirdly, preliminary observations showed that hypertonic solutions can cause an increase in free-cytoplasmic Ca^{++} as monitored by aequorin luminescence. Also, the absence of external Ca^{++} may increase free-cytoplasmic Ca^{++} in *Chironomus.* Neither treatment by itself here was sufficient to cause immediate uncoupling but the combination of both conditions may have raised the free-cytoplasmic Ca^{++} quickly enough to the concentration needed to cause uncoupling.

This work was supported by NIH Grant HD-07098.

Lateral diffusion of photopigments in the outer segments of rods of Bufo marinus. T. P. WILLIAMS, E. F. MACNICHOL, JR., AND H. E. JOHNSON.

The diffusion of unbleached rhodopsin and its relatively long-lived photoproducts can be used to study the fluidity of the disc membrane of rod outer segments. Using a microspectrophotometer which scans once every second, the transverse absorbance changes of single rod outer segments were measured following strong bleaching illumination coincident with the measuring beam or displaced laterally to it. The absorbance of the main peak at 600 fresnels (500 nm) was decreased immediately following a strong 5 sec coincident bleach followed by a marked increase in absorbance over a 30 sec period, indicating inward migration of unbleached rhodopsin. This effect was reversed by a five hour immersion in an identical solution containing 2% glutaraldehyde to stop diffusion. Thus, the increase in density does not appear to be due to the buildup of a late photoproduct such as pararhodopsin. With laterally displaced bleaching light a larger progressive decrease in absorbance of the main peak was observed than with glutaraldehyde, indicating outward diffusion of unbleached rhodopsin. The half time of the effect was about 6 sec for pH 4.0 and 7.2 and somewhat longer at pH 8.0.

After bleaching, there was a marked increase in ultraviolet absorption, presumably by metarhodopsin II, which behaved in a predictable fashion. There was also evidence of a very slow buildup of pararhodopsin (Meta III) with peak absorption near the main peak of rhodopsin. However, the speed of formation of this product was too slow to account for the results observed during a 30 sec period. It is planned to continue these experiments under conditions having well known effects upon membrane fluidity.

Support from the Rowland Foundation and other nongovernmental sources is gratefully acknowledged.

Effects of predation by Polinices duplicatus (*Gastropoda: Naticidae*) *on a sand-flat community.* WENDY I. WILTSE.

The effects of predation by *Polinices duplicatus* on species composition, density, and diversity of the infauna at Barnstable Harbor, Massachusetts, were studied experimentally. In May of 1975 and 1976, 3 × 3 m fenced plots were established on the sand-flat and maintained as snail exclosures and enclosures. Fenced plots and adjacent controls were sampled in August and November of each year, during the season when snails are active.

For the community, number of species (S), evenness (SD), Shannon's index of diversity (H′), and density all decreased with increasing snail density. This was true for both molluscs (prey) and nonmolluscs (nonprey). Intense predation and selective feeding on rarer species resulted in the loss of individuals and species of molluscs from the community. The negative relationship between snail density and diversity of nonmolluscs resulted from a loss of polychaetes and subsequent increased dominance by sipunculans at high snail densities. Spionid polychaetes and other small tube-worms that live and feed near the sediment surface, showed the greatest differences in abundance between exclosures and enclosures. Paired samples from inside and outside of trails made by snails showed that the density of nonmolluscs, particularly spionids, was significantly reduced inside trails. This suggested that bioturbation of sediments by snails plowing through the sand reduced the density, number of species, and diversity of nonmolluscs in snail enclosures.

Unlike space-limited, rocky-shore habitats where predation enhances local species diversity, the activity of *P. duplicatus* lowered the diversity of the sand-flat community.

Studies of dogfish (Mustelus canis) *ocular tissues: lens protein aging and cornea DNA repair.* TERESA YULO AND SEYMOUR ZIGMAN.

Lens proteins of dogfish of increasing age were separated centrifugally into insoluble (I; sedimented at 12,000 × *g*), colloidal (C; sedimented at 100,000 × *g*), and soluble (S; 100,000 × *g* supernatant) fractions. Using SDS polyacrylamide gel electrophoresis, the chains observed in the S fractions of young animals were mainly of 15,000 to 35,000 daltons. Heavier chains were present in the S fractions of older animals. Chains mainly of 15,000 to 35,000 daltons and some of 60,000 to 90,000 daltons, which accumulated with aging, were found in the C fractions. The same chains plus others of 8000 and 105,000 daltons were also found in I fractions, but the levels of heavy chains were greater. Thus, the predominant chains in all dogfish lens protein fractions have molecular weights of 15,000 to 35,000 daltons. Aging of the lens is accompanied by an accumulation of large amounts of heavy, covalently-linked aggregated protein species in the C and I fractions. These aggregates increase light scattering and decrease lens elasticity.

In vitro far-UV light (<300 nm; f-UV)-induced DNA synthesis was measured in cornea and lens epithelia. F-UV-stimulated DNA synthesis occurred in cornea epithelium during the first two hours post-irradiation, when thymidine (tdr) incorporation in irradiated cells exceeded that of dark controls. F-UV light did not stimulate DNA synthesis in lens epithelium. Since little f-UV light reaches lens epithelial cells, reduction of DNA synthesis may be due to the N-UV light present. The results indicate a difference in repair potential of cornea and lens epithelium. *In vivo*, epithelial cells of cornea, but not of lens, would benefit from an f-UV repair system.

This work was supported by NIH (Eye Institute) and N.Y. State (Health Research Council).

The effects of redox dyes on growth and development of sea urchin (Arbacia punctulata) *eggs.* S. ZIGMAN, B. ANTONELLIS AND S. F. ZIGMAN.

The ability of a series of chemically similar dyes with increasing reduction potentials ($-E_r$) to interfere with mitosis in fertilized sea urchin eggs was studied. Addition of these dyes to the sea water in which the eggs were maintained allowed us to observe the effects of varying the levels of electrons that are donated to the eggs as determined by their $-E_r$'s. These dyes then furnished an entire range of concentrations of electrons to influence the eggs.

Only dyes with $-E_r$'s greater than 1.0 very effectively inhibited mitosis at concentrations as low as 10^{-7} M. When mitosis was inhibited by dyes the mitotic apparatus of the eggs still formed, even when total inhibition of cell division resulted. In experiments demonstrating an inhibition of cell division, a great inhibition of the synthesis of DNA (as measured by ^{3}H-thymidine incorporation into DNA) was also observed. Thus, the eggs treated with dyes having high $-E_r$'s did not divide and synthesized DNA at a rate less than 15% of the control rate. Pretreatment of the eggs with effective dyes permanently inhibited both mitosis and DNA synthesis even after free dye removal by extensive washing. The dyes were permanently attached to the eggs since eggs exposed only briefly to the dyes retained the color during extensive extraction with aqueous and organic solvents. Even when added to more advanced sea urchin embryos (*i.e.*, at 8 and 16 cells), active dyes allowed no further development. Sunlight bleached and destroyed the action of the most active dye (#1).

While the mechanism of redox dye action is currently unknown and unexplored, it appears that an excess concentration of electrons can markedly interfere with cell growth and mitosis. This finding may have great importance and possible practical use in circumstances where inhibition of rapid cell growth is desired.

This work was Supported by Eastman Kodak Co.; Sponsor, Paul Gilman, Jr.

Continued from Cover Two

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THE

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FUNCTIONAL MORPHOLOGY AND DEVELOPMENT OF SEGMENTAL INVERSION IN SABELLID POLYCHAETES

Reference: *Biol. Bull.*, **153**: 453–467. (December, 1977)

N. J. BERRILL

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Sabellid polychaetes have exceptional capacities for repeated reconstitution of missing parts anteriorly and posteriorly from any level of the body (Berrill, 1931), even from single segments (Okada, 1934). Although the morphogenetic processes involved are presumably under ultimate genetic control, the developmental processes, as such, remain elusive. The organism, however, is both a present and an historical entity, consisting of primary organizational (phyletic) features and superimposed adaptive modifications and specializations. A clear picture of the phenotype is necessary to the unraveling and understanding of the formative processes. The following account, mainly of *Branchiomma nigromaculata* (Baird) is made with this in mind. Nevertheless, any living organism has its own intrinsic interest as a going concern.

Sabellids live in a mucus-lined tube, with the prostomial crown of tentacles usually extended beyond the distal end of the tube. This complex food-collecting apparatus has been well described by Nicol (1931) for *Sabella pavonina*. Her observations are confirmed for *Branchiomma nigromaculata*, although they are peripheral to the main interest here, which concerns the significance of parapodial inversion of thoracic, compared with abdominal, segments. In sabellids and serpulids the segmental parapodia typically bear both a bristle bundle (setae) and a torus, or ridge with a row of hooks (uncini), which serve for locomotion and anchorage within the tube. The setae and uncini are respectively dorsal and ventral in anterior, thoracic segments, but are respectively ventral and dorsal in all abdominal segments. Why this is so, is the problem, with regard to both development and function.

In the sabellid long known as *Branchiomma vesiculosum* (Montagu), but now commonly referred to as *Megalomma vesiculosum*, the postlarval stage, according to Wilson (1936), elongates by addition of new segments posteriorly that are already thoracic in character, until the full number of eight thoracic segments have been formed. Only then are abdominal-type segments added. He concluded that this procedure is typical of sabellid development, in contrast to serpulid development, where eggs are typically small (about 80 microns in diameter) and form larvae with initially only three thoracic segments, additional thoracic segments being formed later by conversion of abdominal segments. A comparative study of

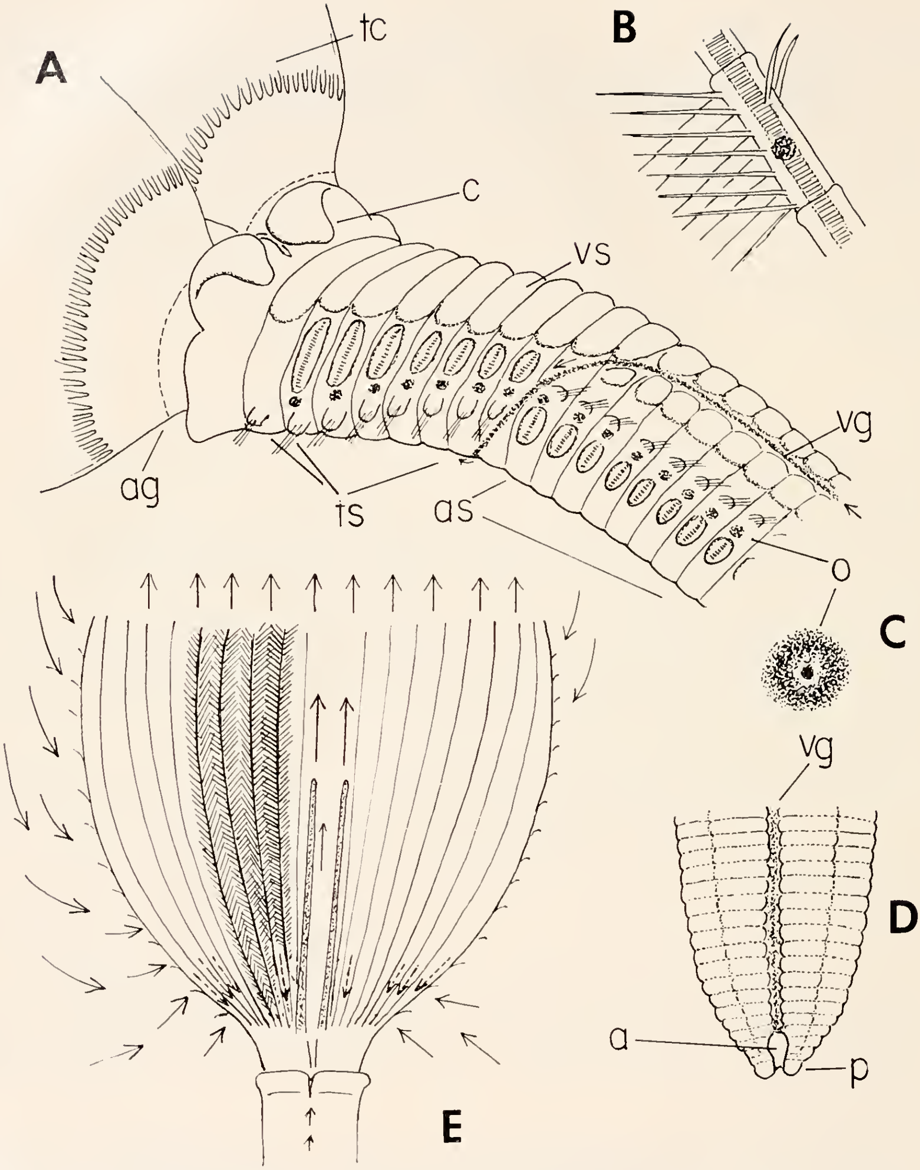

FIGURE 1. External structure of *Branchiomma nigromaculata:* A, anterior region in ventrolateral view showing base of crown of tentacles, collar, thoracic segments, and anterior abdominal segments; the ciliated ventral groove is seen to pass to the dorsal (lower) surface at the junction of thorax and abdomen; uncinigerous tori and setigerous bundles of abdominal segments are inverted compared to those of thoracic segments; B, a segment of a tentacle, showing internal skeleton of choroidal tissue, seven pairs of ciliated filaments, a pair of external processes and one of a pair of ocelli; C, ocellus of segment at same magnification as that of tentacle, showing central cavity; D, posterior region of worm showing ventral groove and pygidium; and E, bilateral crown of tentacles, with pair of palps, with arrows indicating direc-

several sabellid species, however, shows that the developmental procedure varies considerably and can be correlated with the size of the egg.

MATERIALS AND METHODS

A somewhat confusing situation persists concerning the names employed for two well known sabellid genera. According to Johannson (1927) the generic name *Branchiomma* should be used for species having a series of regularly spaced ocelli along each tentacle, or radiole, and that *Megalomma* be substituted for species with a single, large, subterminal ocellus on each radiole. This usage has become widely, but not universally, accepted; for example, it is adopted in Hartman's (1959) *Catalogue of the polychaetes of the world* but not in the 1957 edition of the *Plymouth marine fauna*. The Johannson nomenclature is used here. Thus, species of *Dasychone* become species of *Branchiomma*. Of the species described here, *Branchiomma nigromaculata* (Baird), an unnamed dwarf species, and *Potamethus elongatus* (Treadwell), were studied at the University of Hawaii Marine Institute, Kaneohe Bay, Oahu, Hawaii; while *Potamilla neglecta* (Sars) and *Fabricia sabella* (Ehrenberg) were studied at Boothbay Harbor, Maine. The late Dr. Olga Hartman confirmed the species identifications.

Branchiomma nigromaculata is notable for the ability of individuals removed from their tubes to secrete a new tube immediately, attach to any available surface, and to do so repeatedly. The whole worm becomes encased in a transparent mucoid tube open distally and by a small pore near the posterior end. The activities of the worm within its tube become clearly observable, which is rarely the case in other species. Developing embryos were obtained in this species and in others from natural spawnings, and glass slides with newly settled postlarvae were suspended in sea water in sheltered locations, where rapid growth occurred.

RESULTS

Form and function

An obvious feature in *Branchiomma nigromaculata* is a segmental series of dark pigment patches on both sides of the body, each patch overlying an ocellus. The patch lies between the medial end of the torus and bristle bundles in thoracic and abdominal segments alike, forming a single series (Figs. 1A, 3A). This may be taken as a stable point of reference for all segments. In other species, pigment patches alone may be present, *e.g.*, in *Sabella pavonina*.

A deep, ciliated, midventral groove, dividing a prominent ventral glandular shield into a right and left part in each abdominal segment, extends forward to the thoracico-abdominal junction. From there it passes up the right side of the body to the dorsal surface, and there continues in a less well-defined form to the anterior end of the worm (Fig. 1A). The ventral shield continues forward from the junction as an undivided glandular mass in each segment. The groove is strongly ciliated and carries feces from the anus to a point of discharge dorsal to the mouth. The turn-over of the groove and the parapodial inversion give the illusion that

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tion of currents produced by ciliary activity. Abbreviations are: a, anus; ag, autotomy groove; as, abdominal segments; c, collar; o, ocellus; p, pygidium; ts, thoracic segments; vg, ventral groove; and vs, ventral glandular shield.

the worm is twisted through 180 degrees at the junction between abdomen and thorax.

The tentacular crown of sabellids is light-sensitive, with receptors commonly spaced along each tentacle radiole. In *Branchiomma nigromaculata* they are multicellular, multilenticular ocelli, with up to 29 per tentacle in the largest individual, with tentacle number up to 40 in each half crown. Any rapid change in light intensity, as produced by a moving hand or the approach of a predator, induces the "shadow reflex" resulting in instant withdrawal within the tube. The action is mediated by a pair of giant axons originating in the dorsal cerebral ganglion and extending along the ventral nerve cord the whole length of the body. (In the largest sabellids, namely, *Myxicola infundibulum, Eudistylia vancouverensis,* and *Sabellastarte magnifica,* in fact, when a worm is removed naked from its tube and held in a closed hand out of water, sudden contractions are felt as definite thuds.) The contraction of the body is complete, every segment being shortened anteroposteriorly to maximum degree and at the same time widened to maximum extent. The shortening and widening, however, affects the posterior portion of the body first, so that the body tends to lodge in the tube posteriorly and to be drawn there as a whole (Fig. 2A, F).

As seen within the transparent tube of *Branchiomma nigromaculata,* such contractions are followed by a regular cycle of further action. After a variable period of contraction, the body slowly extends, the anterior end moving forward to take up the slack without much change in the position of the posterior region that previously served as an anchor (Fig. 2B). During the process of re-emergence of the head, viewed from the open-end of the tube, the crown may be seen to undergo one or two full rotations. When fully extended, with crown protruded and collar flush with the distal margin of the tube, the thorax itself becomes anchored, with the remainder of the body now hanging free (Fig. 2C).

Almost immediately following, a variable but small number of peristaltic waves, commencing at the anterior region of abdominal segments, pass down the length of the worm, without any accompanying change in body length as a whole (Fig. 2D). At first the waves pass about halfway along the worm. Successive waves pass further and further, until the whole worm is involved. They serve for irrigation of the tube contents, for at least some respiration, and, in this species, there is some movement of water out through the posterior tube aperture. After the irrigation waves cease, the whole body becomes somewhat drawn up, and almost the entire lateral surfaces become applied to the inner wall of the tube (Fig. 2E).

As already noted, parapodia in sabellids each consist of a bulb carrying a bundle of setae, and a more elongate torus bearing a row of hooks (Fig. 1A, 3A). In *Branchiomma nigromaculata* their operation within a newly formed tube is readily seen. The setigerous bundles serve for "walking" forward and backward, and the uncinigerous tori exclusively for holding position, either for the whole worm or a portion thereof.

In all sabellids examined, large and small, of both subfamilies, as well as in serpulids, the hooks throughout the body all point forward, in thoracic and abdominal segments alike. Individually, hooks are minute and ineffective. In aggregate their holding effect is considerable. In the largest species already mentioned

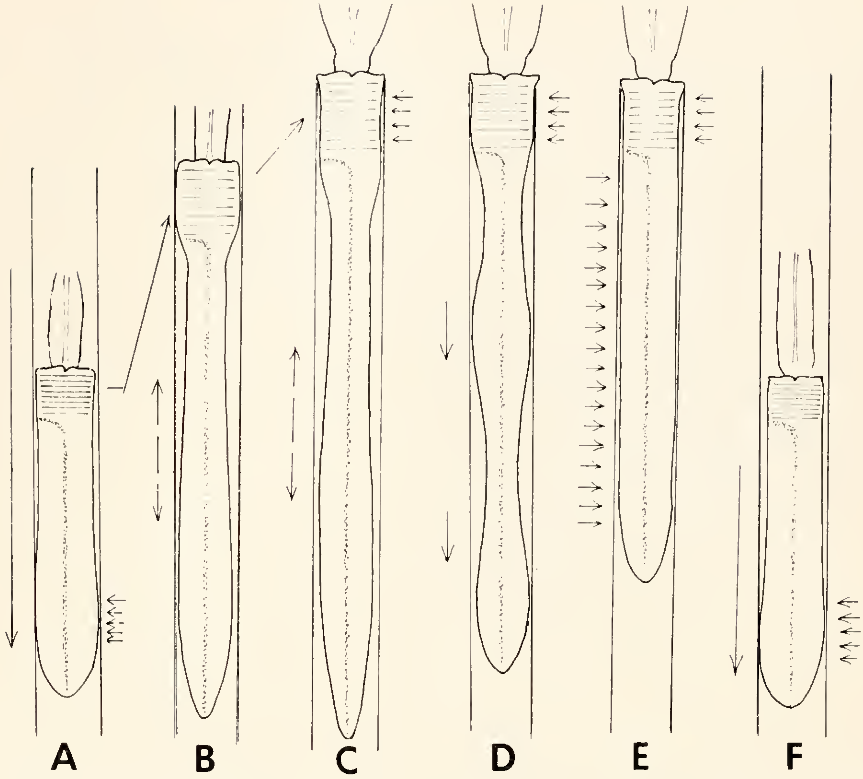

FIGURE 2. Cycle of movement of body of *Branchiomma nigromaculata* within the tube, seen from ventral side (points of anchorage indicated by horizontal arrows; direction of movement by vertical arrows): A, individual fully withdrawn, anchored posteriorly; B, body extending from contracted state; C, body fully extended, and anchored in thoracic region; D, fully extended with peristaltic irrigation waves passing posteriorly along the body from the anterior end of the abdominal region; E, body in full anchored state during stable filter-feeding, *i.e.*, anchored throughout both thorax and abdomen; and F, fully contracted state, induced by external disturbance or shadow initiating giant axon reflex.

they may amount to several thousand. In *Branchiomma nigromaculata* and many other species, the hook has a main fang with several smaller teeth above it, all directed forward (Fig. 3C). The main fang especially is capable of catching in the mucoid inner lining of the tube. In the resting, extended state the parapodia are pressed against the wall of the tube, and the tori with their rows of hooks become engaged and resist any tendency of the worm to slip forward.

During the resting, feeding state, firm anchorage of the anterior thoracic end of the worm, with collar flush with the rim of the tube, is required. Relating to this, the tori of the thoracic segments are longer more anteriorly, with correspondingly larger numbers of hooks. Nevertheless, the capacity of the hooks alone to securely anchor the body, or a part thereof, is limited.

Thoracic setal bulbs of most sabellid species carry two series of setae of somewhat different character. In *Branchiomma nigromaculata* an upper series has

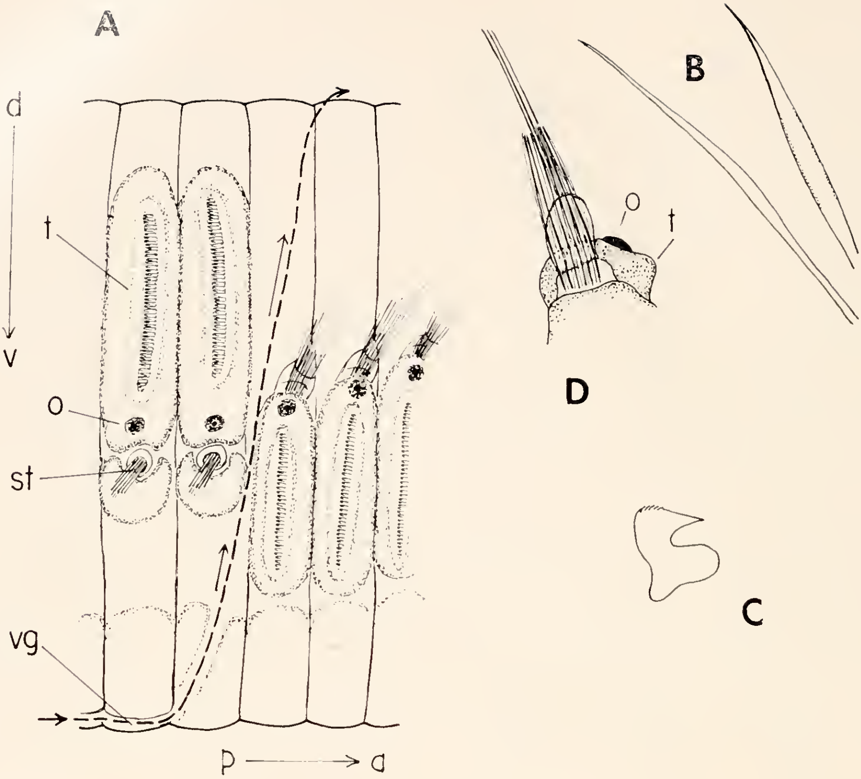

FIGURE 3. Parapodial structure and orientation in thoracic and abdominal segments of *Branchiomma nigromaculata:* A, abdominal segments with ventral setigerous bundle in mid-lateral region, and torus with hook row and ocellus in dorso-lateral region; and thoracic segments with ventro-lateral torus with hook row and ocellus, and setigerous bundle directed dorsally and forward; B, setae, typical of thoracic segments; C, uncinus, typical of both abdominal and thoracic segments, with hook pointing anteriorly; and D, abdominal para-podium in ventral view showing setigerous bundle consisting of fleshy lobe with about a dozen regular setae and several relatively long setae emerging through it, together with ventral end of torus bearing the segmental ocellus (in silhouette) projecting directly outward from mid-lateral region of segment. Abbreviations are: d-v dorsoventral; o, ocellus; p-a, antero-posterior axis, anterior to the right; st, setigerous bundle; t, torus; and vg, ventral groove.

short, tapering winged tips and more elongated striated shafts, while a lower series has broader striated wings, serrated at the edges (Fig. 3B). In abdominal segments the setae occur in small tufts, the upper setae with much elongated tips and the lower with distinct wings. In other species the differences may be greater, with one kind of seta more slender and the other more paddle-like below the fine tip. Such are the actual instruments employed to maneuver the body forward or backward or to rotate the anterior end. In operation the more or less wingless, slender setae may penetrate the mucoid lining of the tube to a considerable extent, while the winged setae penetrate only to the extent of the tip beyond the wings.

Muscles inserting on the setal bulbs move the base of the bundles and, thus, cause the setae to exert leverage against the tube lining in which they impinge, as in the use of oars. As a rule, and *Branchiomma nigromaculata* is no exception, the thoracic bundles with their associated segmental muscles are somewhat larger and more powerful than the abdominal bundles, although this difference is offset by the relatively small number of thoracic segments. The thoracic setigerous bundles function primarily in walking, or levering the body forward. When at rest, the thoracic bundles point dorsally, outward and slightly forward from the segmental body wall (Fig. 3A). When the parapodial and segmental longitudinal muscles contract, the setae are protruded and engage the lining of the tube, and an effective backward stroke is produced, moving the segment forward. The parapodium then swings forward to the original position and the setae are released.

A stepping wave of contraction of the segmental longitudinal muscles passes down the whole worm, the two sides of the worm being out of phase with one another. This results in a spiral advance of the worm and a twisting of the anterior, thoracic region relative to the abdominal region which is free to unwind. The forward motion of the worm resulting from the action of the thoracic segments is resisted to some extent by the forward-pointing hooks, which are seen to catch in the tube lining and to be pulled free. The thoracic setae also serve to anchor against movement of the body posteriorly, particularly when the worm is not in active movement, for when anchored anteriorly and hanging freely in the tube, it cannot be pulled back through the posterior end of a cut tube without pulling the worm apart, usually. On the other hand, stimulation of the posterior end of a worm causes emergence from the front end of the tube, but rarely farther than the posterior end of the thorax.

Abdominal setigerous bundles are employed for walking backward. Abdominal bundles move in continuous series with the thoracic bundles but on close scrutiny do not appear to do any effective work with regard to forward progression. At rest, the setae extend outward, ventrally, and backward from the segmental wall (Fig. 3A). In walking the body, each setigerous bundle engages in the tube lining and strokes forward, causing the segment to move backward. At the end of the stroke the setae are disengaged. The cycle is similar to that of thoracic setigerous bundles but is concerned with differently oriented structures. In *Branchiomma nigromaculata* between fifty and a hundred abdominal segments are thus involved. The overall activity causes the worm to migrate to the posterior end of the tube, which is seen in circumstances that induce withdrawal without triggering the shadow reflex. In some species, such as *Sabella pavonina,* which may have tubes imbedded in mud to a depth of two feet, with up to one foot of tube standing above the mud, descent to the lower end of the tube is necessary at times in order to dredge up tube-building material. However, the much more vital function of the reversed action of the abdominal setae appears to be the capacity of the abdominal setigerous bundles, individually and collectively, to resist strongly any forward pull exerted on the body, greatly augmenting the combined effect of the forward-pointing hooks of all the segments.

Segmental transformation

The eggs of *Branchiomma nigromaculata* are mostly from 130 to 135 microns in diameter and appear well-packed with yolk granules. Unlike the trochophore

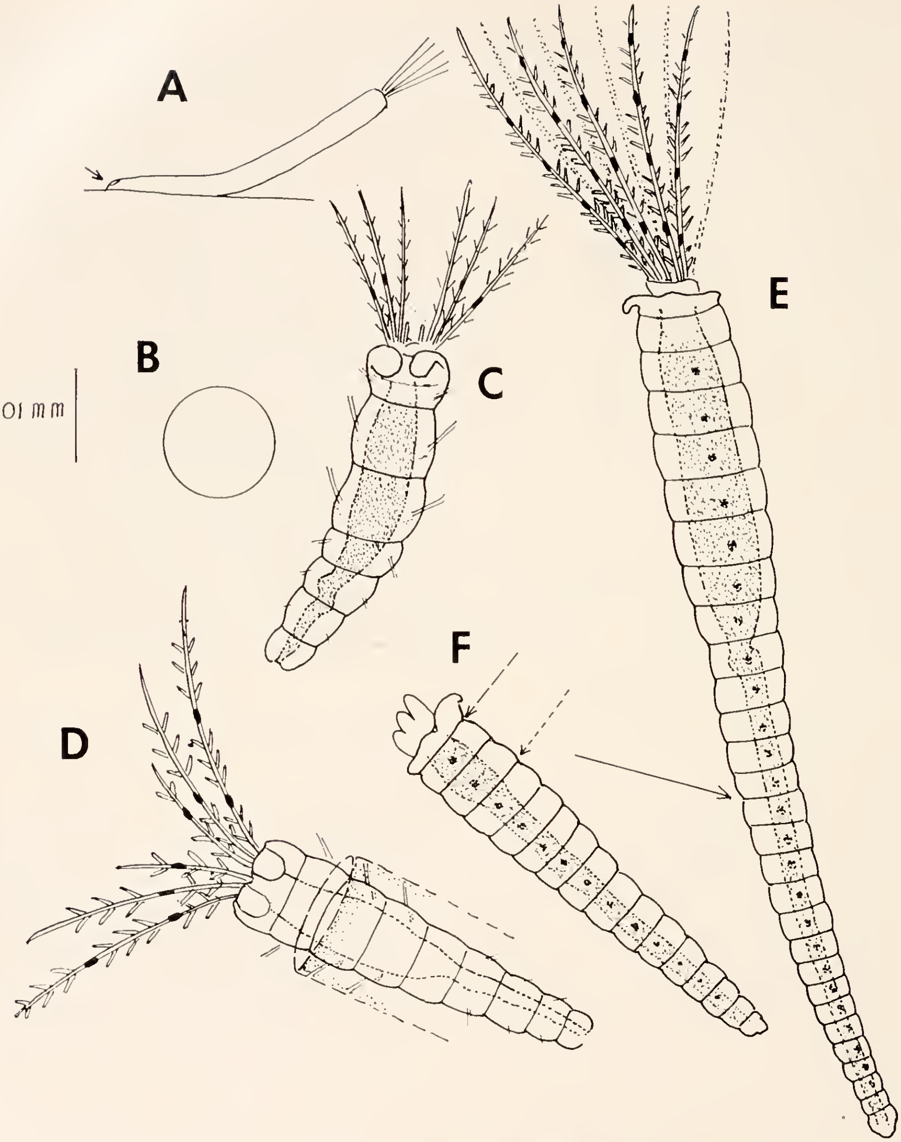

FIGURE 4. Postlarval development of *Branchiomma nigromaculata* (parapodial setae and hooks are not shown in E and F): A, attached juvenile, 7–10 days, showing characteristic elevation of anterior part of the tube, and posterior aperture; B, egg, at same magnification as juvenile stages; C, initial stage (first week) exhibiting bilobed collar (ventral) with 3 thoracic and 3 abdominal segments; D, somewhat older juvenile (10 days) with 4 thoracic and 3 abdominal segments; E, fully formed juvenile, from side, with 8 thoracic and 19 abdominal segments; and F, abdominal piece cut from stage in E, after five days, showing a regeneration of new tentacles, collar, and beginning of thoracic transformation of two most anterior abdominal segments. The intestine is indicated by dotted lines and the wide thoracic region is seen to be coextensive with the thorax. Pigment patches in E and F represent ocelli in lateral body

larvae of serpulids, which develop from an egg of about 80 microns in diameter, the free-swimming sabellid trochophore stage does not feed, and a functional planktonic phase is brief. Newly-settled metamorphosed larvae immediately secrete a delicate tube of mucus, which has a small posterior opening and an elevated anterior part (Fig. 4A). The completely metamorphosed and functioning postlarval stage exhibits three tentacular radioles on each side, with lateral filaments (pinnules) and, on two tentacles, a single eye spot (Fig. 4C). At this stage the body consists of the collar, three thoracic segments, three or four abdominal segments, and the terminal pygidium (Fig. 4C). Following the addition of one or two more abdominal segments, the foremost abdominal segment loses its setae and hooks (Fig. 4D), and later acquires new setae and hooks in the inverse or thoracic disposition. This procedure is repeated until altogether eight thoracic segments are present, five of which have resulted from successive transformations of the most anterior abdominal segments (Fig. 4E).

During this transformative period of juvenile development, the number of tentacles on each side, in each half crown, increases from three to five, each tentacle now possessing four ocelli. An ocellus also appears on the body wall of each side of each segment, located between the setae and hooks in thoracic and abdominal segments alike (Fig. 4E). It is, however, absent on the segment adjoining the collar.

Also evident, in such small transparent juvenile stages, is that the thoracic gut extends posteriorly coexistent with the posterior extension of the thoracic parapodial, segmental character (Fig. 4C, D, E). In the case of the individual shown in Fig. 4E, the posterior abdominal region consisted of fifteen abdominal segments, which, when cut off, regenerated a new head consisting of crown and collar (Fig. 4F), with evidence (enlargement of the gut) of the two anterior segments transforming into thorax. The anterior regenerative-reorganizing capacity typical of sabellids is accordingly present at a somewhat surprisingly early stage of growth.

The progressive conformation of thoracic gut and parapodial character is more dramatically shown in Figure 5. In this species, at least, gut transformation appears to precede conversion of abdominal to thoracic parapodial type, inasmuch as full enlargement of the gut appears posteriorly, before thoracic setae and hooks are visible in the respective segments. Parapodial transformation is already underway in such segments, however, as indicated by the loss of the previously present abdominal parapodial structure (Table I). The question whether thoracic gut extension truly precedes and perhaps induces parapodial conversion cannot, therefore, be determined from these observations. What is evident is the successive manner of transformation of segments along the anteroposterior axis. Moreover, this developmental procedure in *Branchiomma nigromaculata,* which is very similar to that reported for serpulids, and different from that of *Megalomma vesiculosum,* suggests that some cause other than familial inheritance is responsible for the differences.

In addition to the relatively detailed accounts of the contrasting postlarval

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wall of each segment except collar, first thoracic segment, and the posterior pygidium. Scale applies to figures B–E.

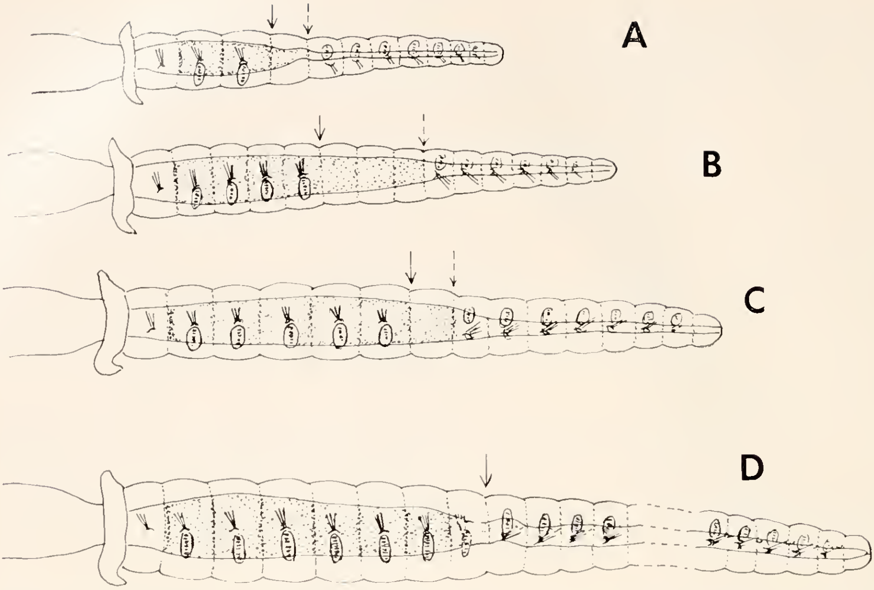

FIGURE 5. Correlation between segmental parapodial transformation and thoracic gut extension in juvenile *Branchiomma nigromaculata:* A, juvenile with 3 thoracic, one transforming and 7 abdominal segments; B, juvenile with 5 thoracic, 3 transforming, and 6 abdominal segments; C, juvenile with 6 thoracic, one transforming, and 7 abdominal segments; D, juvenile with 8 thoracic, none transforming and 19 abdominal segments. Thoracic setae are dorsal, hooks ventral; abdominal setae are ventral, hooks dorsal. In the transforming region between thoracic and abdominal segments, the abdominal segments lose abdominal parapodial structures as the thoracic gut extends at the expense of abdominal gut. Solid arrow indicates posterior limit of fully transformed segments, broken arrow indicates posterior limit of transforming segments.

development of *Megalomma* [*Branchiomma*] *vesiculosum* given by Wilson (1936) and *Branchiomma* [*Dasychone*] *nigromaculata* presented here, the little that is known concerning postlarval development in five other species is somewhat illuminating.

In *Potathemus elongatus*, a species with eggs of about the same size as those of *Branchiomma nigromaculata*, the newly settled postlarva (Fig. 6A) has two segments with thoracic type parapodia and three abdominal, with thoracic gut extending through the first two segments and partially through the third. In the dwarf species, as previously recorded (Berrill, 1977), and seen here in Fig. 6B, five thoracic segments are initially present, with four or five abdominal segments. Eggs are approximately the same size as in the two species just mentioned (Table II).

In the remaining species the egg size is much larger, more than three times greater in volume, of the order of 200 microns or more in diameter. In all three species the full number of thoracic segments form directly, with abdominal segments being added posteriorly later. In *Potamilla neglecta* (Fig. 6C) new abdominal segments are successively added during later growth until sixty to seventy have been formed.

Postlarval development in *Potamilla neglecta,* however, shows another very significant feature. The extension posteriorly of the thoracic gut lags far behind the establishment of thoracic parapodial character. In *Sabella microphthalma,* the full number of thoracic segments again is attained directly, with an indefinite number of abdominal segments subsequently added (Kerby, 1971). In *Fabricia sabella* the exceptionally large and yolky egg develops the full number of thoracic segments directly, while yet an embryo within the parental tube (Fig. 6D). Abdominal segments are added later posteriorly, but only to a total of three (Fig. 6E).

Discussion

In sabellids, and in the closely related but much more specialized serpulids, the thoracic region, as defined by the dorso-ventral orientation of the setigerous and uncinigerous units of the parapodia, is generally limited to about eight segments. These segments are solely responsible for forward locomotion in the tube, and may represent the minimum number compatible for effective progression (*cf.,* Gray, 1939, for analysis of stepping waves of four to eight segments in *Nereis*). In other polychaetes, including the tubicolous terebellids (Thomas, 1940), the thoracic, dorso-ventral orientation of the parapodia extends posteriorly from the head throughout the length of the body. In no case is there any inversion as seen in sabellids and serpulids; all setigerous segments are employed in walking forward.

Apart from the particular, highly differentiated structure and function of the crown as a feeding organ, the outstanding morphological feature of sabellids is accordingly the inversion of the parapodial structure in so-called abdominal segments, together with the relatively great extent of the inverted segments, which may exceed 300 in some species, *e.g.,* in *Sabella pavonina* and *Sabella spirographis.*

Sabellids suffer severe predation by fish in spite of protective mechanisms and behavior, unlike serpulids which are encased in a cemented calcareous tube and can lose only parts of their tentacles. *Sabella pavonina,* for example, exists in huge populations on the northern Atlantic Continental Shelf and parts of them are found in large numbers in the stomachs of bottom-feeding fish. In any large sample of apparently intact worms dug from lowtide mud flats, about 30 per cent show internal evidence of reconstitution. This situation has persisted at least since the upper Silurian, inasmuch as fossil serpulids are known from that period

Table I

Postlarval development of Branchiomma nigromaculata *(each category represents the range based on about 25 individuals).*

Number of tentacle radioles	Eyespots per radiole	Setigerous thoracic segments	Transforming segments	Abdominal segments	Total number of segments
2 + 2	1 – 2	1 – 4	1 – 2	3 – 8	7 – 16
3 + 3	2 – 3	5 – 6	2 – 3	5 – 11	12 – 20
5 + 5	3 – 4	6 – 8	0 – 2	7 – 19	13 – 29
14 + 14	11 – 15	8	0	83	91
40 + 40	25 – 29	8	0	103	111

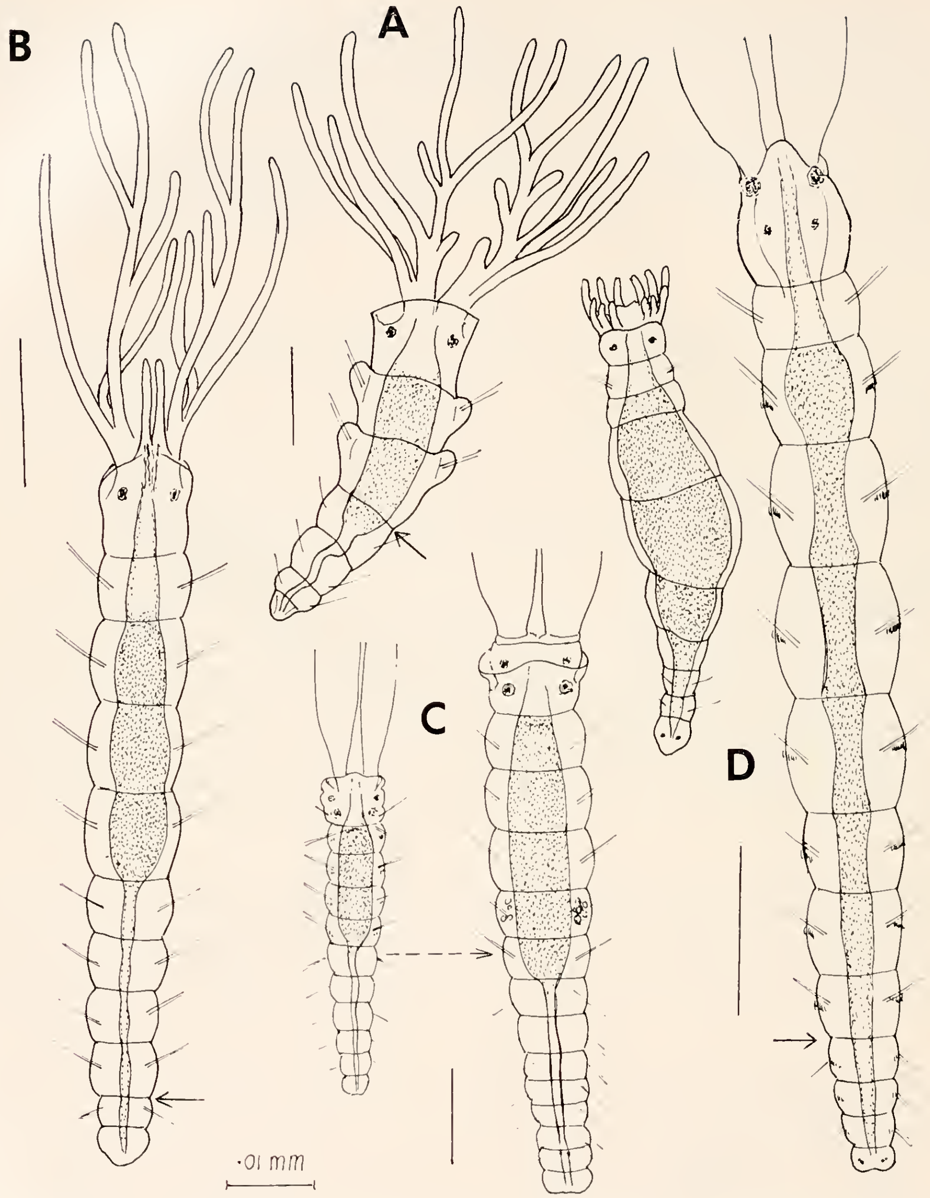

FIGURE 6. Settled juvenile (postlarval) stages of various sabellid species, showing correlations between number of thoracic segments initially formed and the size of the egg. Straight lines indicate diameter of respective eggs (see Table II). A, *Potamethus elongatus,* small egg size and postlarva with two primary thoracic segments and three abdominal segments, with thoracic gut already extending into first abdominal segment; B, *Potamilla neglecta* showing large egg size and postlarva with eight primary thoracic segments and one abdominal segment, with posterior extension of thoracic gut lagging relative to thoracic character of the body wall; C, egg size, first juvenile and incipiently sexually mature stages of a dwarf species, showing

Table II

Egg size and number of initial thoracic segments.

Species	Egg diameter in microns	Number of thoracic segments initially formed
Branchiomma nigromaculata	135	3
Potamethus elongatus	110–150	3
Dwarf species (Berrill, 1977)	140	5
Megalomma vesiculosum (Wilson, 1936)	150	8
Fabricia sabella	200	8
Potamilla neglecta	200	8
Sabella microphthalma (Kerby, 1971)	250	8

and serpulids have undoubtedly evolved from a sabellid stock. The filtration-feeding crown of a sabellid, which is large relative to the size of the body (Dales, 1957), is necessarily ostentatiously displayed, and a considerable part of the worm in the distal part of the tube is likely to be bitten off, in spite of a fast shadow reflex, although autotomy of the crown may often be a saving grace.

The conversion of the greater part of the worm from forward-moving to backward-moving locomotory structure in each segment, with the concomitant digging-in of the parapodial heels, so to speak, virtually ensures that some of the more posterior portion of the worm will be left behind in the tube when the distal part is pulled from the front. This, combined with the extraordinary capacity, in sabellids, of abdominal segments, at all levels of the body, to regenerate a new head, and for the most anterior of the surviving abdominal segments to reorganize into thoracic-type segments, guarantees that a new individual forms from the one that was pruned.

From the observations on segmental transformation in *B. nigromaculata* and other species, it is evident that there are two dominant features in postlarval development and growth. Posterior extension of the body occurs by means of successive formation of abdominal segments from a zone of growth immediately anterior to the terminal pygidium. Thoracic segment character extends posteriorly from the metamorphosed larval head. Both processes vary from species to species. The final number of definitive abdominal segments, apart from any that may have become transformed to thoracic, ranges from three to about three hundred. The final number of thoracic segments is typically eight, but these may be all formed directly, or a smaller number may be formed directly and the remaining thoracic segments added through transformation of abdominal segments. Altogether these phenomena support the concept of two morphogenetic agencies working from

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four primary thoracic segments and five abdominal segments in the juvenile stage, and the conversion of the first abdominal segment to thoracic both with regard to the gut and the parapodia (broken arrow); and D, egg size, advanced embryo, and fully developed stages of *Fabricia sabella,* showing heavily yolky endoderm of embryo, and direct formation of eight thoracic segments, with terminal three abdominal segments (the complete number) added to form the final condition.

opposite ends toward the middle, a concept long evident from experimental studies of regeneration and reorganization.

The extent of posterior abdominal growth, most simply expressed by the total number of abdominal segments successively produced, is a variable independent of other aspects of growth, and constitutes a major developmental problem. The nature of the "morphogenetic field" responsible for the imposition of thoracic character anteriorly is also a fundamental problem, but at least the variability expressed in this study indicates a correlation between egg size and initial extent of such a field of influence, *i.e.,* the larger the egg, the larger the larvae, particularly the anterior region. This in turn has a correspondingly greater dominance over posterior structure in the process of development.

Summary

The structure and function of the external features of sabellid polychaetes are described, with special reference to the species *Branchiomma nigromaculata.* Form and function particularly are analyzed with reference to the ability of such worms to survive radical mutilation by predators. In addition to adaptive features already known, namely, "shadow reflex" withdrawal involving giant axon stimulation, autotomy of the crown, regeneration of a head, and reorganization of a new thorax from old abdominal segments, the crucial structural modification in the intact worm is the parapodial inversion of all segments posterior to the first eight or so anterior segments. This enables the parapodia to dig into the inner wall of the tube in such a way that a predator cannot pull an entire worm out of the tube. A posterior part is inevitably left behind to reconstitute a complete individual.

During postlarval growth, in several species, namely, *Potamilla neglecta, Sabella microphthalma,* and *Fabricia sabella,* all with comparatively large eggs, all thoracic segments (typically eight) are formed before any abdominal segments are produced. In other species, *Branchiomma nigromaculata, Potamethus elongatus,* and the dwarf species, all with comparatively smaller eggs, less than the full complement of thoracic segments are initially produced, and the remainder are subsequently formed by successive conversion of adjoining abdominal segments, in accordance with the procedure typical of the development of the small eggs of most serpulid polychaetes.

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THE EFFECTS OF SELECTED CELL OSMOLYTES ON THE ACTIVITY OF LACTATE DEHYDROGENASE FROM THE EURYHALINE POLYCHAETE, *NEREIS SUCCINEA*[1]

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The most commonly studied intracellular osmolytes of animals are the major inorganic ions, namely potassium (K), and, occurring at lower concentrations, sodium (Na) and chloride (Cl). In muscle tissue, which in most animals is the preponderant cell type in terms of body volume, the intracellular concentrations of these ions, particularly K, are relatively constant despite wide variations in total cell osmolarity (Burton, 1968, 1973, Figure 1). In the euryhaline polychaete, *Nereis succinea*, for example, the total intracellular concentration of these three ions ranges from 315 mM in animals adapted to 100% sea water (SW) to 147 mM in animals adapted to 10% SW. Potassium ion concentrations over this salinity range are decreased only in response to incomplete cell volume regulation with osmotic dilution (Freel, Medler and Clark, 1973).

Since the plasma or hemolymph ion content of a wide range of animals varies from about 300 mOsmol for freshwater species to >1000 mOsmol for marine species (Prosser, 1973), the intracellular osmotic activity of species with high body fluid osmotic pressures must be provided by solutes other than inorganic ions. Low molecular weight nitrogenous solutes comprise a major fraction, up to 0.6 M, of the intracellular solutes of marine invertebrates (Shaw, 1958; Jeuniaux, Duchâteau-Bosson and Florkin, 1961; Robertson, 1961; Awapara, 1962; Clark, 1968a; Freel *et al.,* 1973). Aside from the phosphagens, which are relatively constant at all osmotic dilutions and comprise some 64–82 mM in *Carcinus* (Shaw, 1958) and 12–14 mM in *Nereis* (Freel *et al.*, 1973), the remaining nitrogenous solutes are mainly amino acids, taurine, betaine, trimethylamine oxide (TMAO) and, in vertebrates, also urea (Lutz and Robertson, 1971; Robertson, 1975, 1976). Among euryhaline species, the concentrations of these solutes are regulated during adaptation to reduced environmental salinities (Shaw, 1958; Jeuniaux *et al.,* 1961; Virkar, 1966; Clark, 1968b; Freel *et al.*, 1973; Schoffeniels, 1976). So far, however, no explanation has been proposed for the function of these organic nitrogenous osmolytes, the maintenance of which at such high intracellular levels, in preference to readily available inorganic ions, must represent a significant energy expenditure by the organism.

Neutral salts have long been known to differ in their effects on the conformation of a wide variety of macromolecules including globular proteins and enzymes, fibrous proteins such as collagen, model polypeptides, and nucleic acids. In the Hofmeister or lyotropic series, cations such as NH_4^+ and $(CH_3)_4N^+$, and anions such as $SO_4^=$

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and CH_3COO^- favor the native, functional state; the environmentally common ions, such as Na^+, K^+ and Cl^- are less favorable; and ions such as Ca^{++}, Mg^{++}, I^-, ClO_4^- and SCN^- are distinctly deleterious at comparable ionic strengths (von Hippel and Schleich, 1969). As shown in Figure 1, the functional groups on the nitrogenous solutes accumulated by marine invertebrates are comparable to NH_4^+ (amino acids, taurine); to $(CH_3)_4N^+$ (TMAO, betaine); to $SO_4^=$ (taurine) and to CH_3COO^- (amino acids, betaine). One can therefore postulate that high intracellular concentrations of K^+, Na^+ and Cl^- would be injurious to macromolecular function and that these organic solutes serve a protective, or at least substitutive role in animals with blood osmotic pressures significantly greater than 300 mOsmol.

Most studies of specific ion effects on macromolecular conformations have been concerned with gross conformational changes, such as helix-coil transitions, that are observed only at far greater temperatures and ionic strengths (von Hippel and Schleich, 1969) than those experienced by most osmoconforming euryhaline invertebrates. It is therefore of interest to know whether lower concentrations of lyotropically less favorable solutes can effect grossly undetectable, yet functionally significant conformational changes in biological macromolecules. Some evidence that this is indeed the case exists in the observation by Warren, Stowring and Morales (1966) and by Warren and Cheatum (1966) of significant inhibitory effects of relatively low (0.5 to 1.0 M) concentrations of biologically prevalent salts, such as NaCl and KCl, on the activities of a number of vertebrate enzymes. Such observations have been confirmed recently by studies of neutral salt effects on enzymes of varying origin (Borowitzka and Brown, 1974; Somero and Low, 1977; Somero, Neubauer and Low, 1977).

The experiments described here were undertaken to test the following hypotheses: first, that an intracellular macromolecular of a marine invertebrate is indeed susceptible to functional inhibition by concentrations of those neutral salts found in the animal's environment and its extracellular fluid; secondly, that lyotropically more favorable salts, such as ammonium sulfate, or nitrogen-containing organic solutes with comparable functional groups, such as amino acids or TMAO, are innocuous to macromolecular function; and thirdly, that this latter group of molecules can offset the effects of functionally deleterious solutes. Examination was made on the functional behavior, in the presence of various solutes, of a representa-

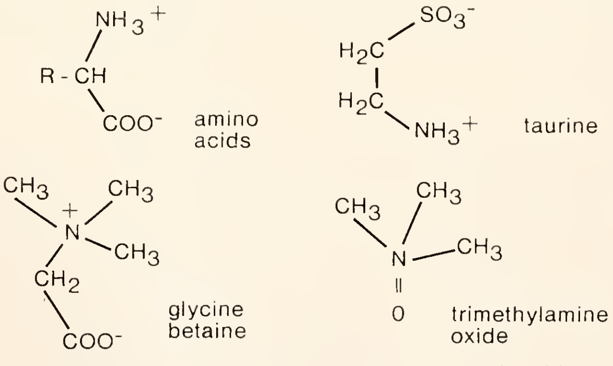

FIGURE 1. Formulas of intracellular nitrogenous solutes commonly found in marine invertebrates.

tive macromolecule, lactate dehydrogenase (LDH) (EC 1.1.1.27) obtained from the body wall tissues (mainly muscle) of a euryhaline polychaete, *Nereis succinea,* adapted to a wide range of salinities. This species is an osmoconformer at salinities greater than 30% SW and a partial regulator at lower salinities (Oglesby, 1965).

MATERIALS AND METHODS

Collection and acclimation of animals

Specimens of *Nereis succinea* were collected from the Colorado Lagoon, Alamitos Bay, Long Beach, California between June, 1971 and January, 1973. They were maintained at 15 ± 2° C, without food, in artificial sea water, salinity 34.8‰, of the following composition: Na, 487.2 mM; K, 9.0 mM; Ca, 9.1 mM; Mg, 48.4 mM; Cl, 556.1 mM; SO_4, 25.5 mM; and HCO_3, 2.1 mM. Lower salinities were obtained by dilution with distilled water. Animals were adapted stepwise through 10% intervals of sea water dilution, remaining at least three days at each concentration and at least one week at the final acclimation salinity before being used. No animals were kept more than two months.

Enzyme preparation

In general, large specimens were preferred for taking tissue samples, but fully mature females and heteronereids were excluded. Pooled tissues were taken from several animals adapted to each of the following concentrations of sea water: 100%, 75%, 50%, 35% and 20%. After removal of gut and parapodia, the body wall tissue was rinsed, blotted and weighed. From dry weight analyses on subsamples, appropriate amounts of 0.25 M sucrose were added to the weighed tissues to give a final dry weight of 21.3 μg per milliliter of homogenate prior to centrifugation. Following homogenization, supernatants were obtained by centrifugation at 22,000 × g for 30 minutes. All procedures were carried out at 4° C or less. Each supernatant was pipetted into several small vials and stored at −10° C until use. Although a single freeze-thaw cycle had no effect on LDH activity, repeated freezing and thawing resulted in significant loss of activity and was avoided.

Homogenates from animals collected at different times tended to vary in absolute activity. Although the cause of this variation is unknown, it did not appear to affect the conclusions reached in this study, since the relative effects of assay variables (pH, ionic strength, solute composition) on activity remained constant from batch to batch.

Enzyme assay

The rate of pyruvate reduction by NADH was assayed by following the decrease with time in absorbance at 340 nm, using a Cary Model 15 recording spectrophotometer. In 1.04 ml of solution, the final composition was: 1.0 ml of 1.44×10^{-4} M reduced coenzyme dissolved in a selected buffer with or without added osmolyte; 0.04 ml of varying concentrations of sodium pyruvate; and 2 μl of supernatant containing LDH. The reaction was initiated by the addition of pyruvate, following five minutes thermal equilibration during which any endogenous substrate was removed. The initial, linear (zero-order) change in absorbance was measured at all substrate concentrations, and there was a linear increase in the reaction rate with an increase in enzyme concentration.

During preliminary studies, it was found that LDH in the absence of added osmolytes exhibits hyperbolic kinetics with increasing pyruvate concentration. Substrate saturation occurs at about 5×10^{-3} M pyruvate and slight inhibition was observed at 5×10^{-2} M. A concentration of 10^{-2} M pyruvate was therefore used to obtain maximum reaction rates. The optimal NADH concentration is 1.38×10^{-4} M. All assays reported here were conducted at room temperature, and no thermal inactivation was observed up to 30° C.

Data are expressed either as change in optical density/minute per 2 μl of supernatant (the equivalent of 0.043 μg dry weight of tissue); or as rates relative to a control rate defined as the reaction velocity in the absence of added osmolytes. All comparative results were obtained at a single, constant temperature, using the same enzyme preparation.

Sodium pyruvate and NADH were obtained from Sigma Chemical Company. NADH was freshly prepared within four hours of use. All salts were reagent grade and nitrogenous solutes were A grade. TMAO was synthesized for us in the laboratory of Dr. Edward Grubbs, Chemistry Department, San Diego State University. Sorenson's buffers were prepared using sodium and potassium phosphates in a ratio of 1:3, to approximate intracellular ratios of these cations.

RESULTS

Effect of buffer composition and pH

The optimum pH for LDH activity was determined in duplicate on homogenates from worms adapted to 100%, 75%, 50%, 35% and 20% SW. LDH supernatants obtained from *N. succinea* adapted to 100% sea water are designated as "100%" LDH; LDH from worms adapted to 20% sea water as "20%" LDH; and so on. Two types of buffer were utilized for determining optimum pH: Sorenson's phosphate buffer and tris-hydroxymethyl amino methane hydrochloride buffer (TRIS). Measurements were made over the pH range of 6.0 to 8.0, using zero order (saturation) kinetics.

The effects of pH, buffer composition and buffer strength on LDH activity from animals adapted to various salinities are shown in Figure 2. In both Sorenson's and TRIS buffers, there is no sharply defined pH optimum for any of the enzymes. The absolute amounts of enzyme are not identical for the two buffers because the homogenates were prepared from two different collections of worms. It is interesting that, in both cases, LDH activity per 0.043 μg dry weight of homogenate is considerably higher for osmoregulators (20% LDH) than for osmoconformers. This cannot be explained by the relatively small effect of amino acid loss during osmotic dilution (Freel *et al.*, 1973) on the dry weight composition, and suggests either an increased synthesis of the same enzyme or, perhaps, synthesis of a new isozyme by osmoconforming worms.

The effect of TRIS buffer at strengths ranging from 0.001 to 0.1 M on a single batch of "100%" LDH at pH 7.0 is compared with that of 0.03 M Sorenson's buffer and 0.1 M trimethylamine (TMA) and triethylamine (TEA) in Figure 2c. There is no difference between the three organic buffers. Increasing organic buffer

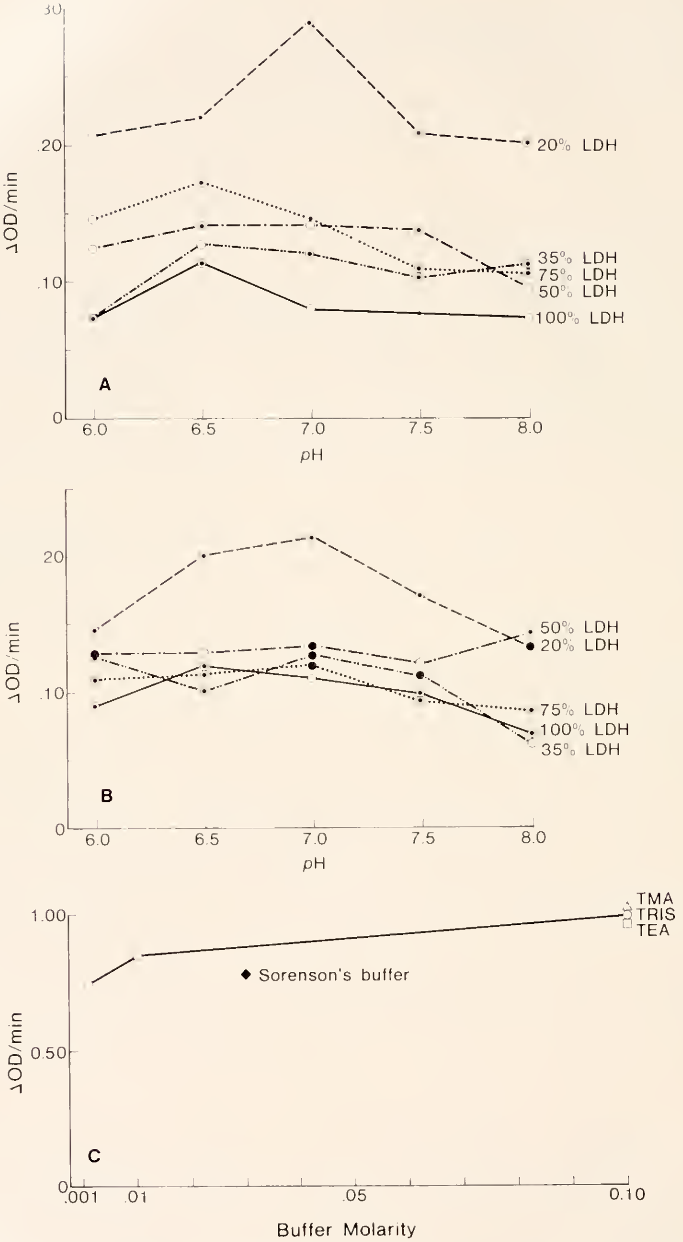

FIGURE 2. The effect of pH, buffer composition and buffer strength on LDH activity: effect of pH in (a) 0.03 M Sorenson's buffer and (b) 0.1 M TRIS buffer; effect of buffer strength (c) on "100%" LDH activity at pH 7.0. Symbols for replicate agreement, which apply to (a) and (b), are: circle with dot, two replicates differ by >20%; filled circle, two replicates differ by >10%; open circle, two replicates differ by <10%; and dot, single determination.

strength increases activity, and Sorenson's buffer appears to be slightly less favorable than TRIS.

All experiments on the effects of added osmolytes were carried out at a pH of 7.0, near the middle of the pH optimum for all homogenates, using organic buffer (TRIS, TMA or TEA) at 0.1 M; this relatively high concentration was chosen to eliminate the effects of small deviations in pH, and to buffer certain strongly ionizing organic osmolytes, particularly taurine. Because the pH optimum studies suggested that "20%" LDH might be a different isozyme from enzymes obtained from osmoconforming worms, this enzyme was studied together with "100%" LDH in many of the subsequent experiments.

Effects of neutral salts

Three cations and three anions were combined in all possible ways, at 0.1, 0.25 and 0.5 M, a range which brackets both the normal intracellular ion concentration and that of full strength sea water. Potassium, sodium and chloride were chosen because they are ions prevalent in cells, body fluids or sea water; ammonium, sulfate and acetate, for their predicted lyotropically favorable effects. Assays were run at saturating substrate concentrations. Data are presented for both cationic and anionic series (Figs. 3 and 4) to permit separate analysis of positive and negative ion effects. Each point is an average of two runs, and the bars represent the range.

It is evident that, as the concentration of salt increases, inhibition becomes greater as compared to the activity in a buffered solution alone; both cations and anions play a role in inhibiting the enzyme. Only in the case of ammonium sulfate on "100%" LDH is there an absence of severe inhibition at high (0.5 M) salt concentration. Other ammonium salts have similar effects on both the "100%" and "20%" enzymes. Sodium ions and especially potassium ions are highly deleterious for "100%" LDH, even at low (0.1 M) and intermediate (0.25 M) concentrations, whereas "20%" LDH is less affected by these ions except at high (0.5 M) concentrations. Neither acetate ions nor sulfate ions are capable of significantly offsetting the inhibitory effects of these cations on "100%" LDH.

Effects of nitrogenous osmolytes

A number of neutral amino acids, taurine, betaine and TMAO were tested for their effects on "100%" and "20%" LDH activity, at concentrations up to 0.5 M where solubility permitted. One to three replicates were run at each concentration, again using saturating substrate concentrations and 0.1 M TRIS buffer.

The effects of neutral amino acids are shown in Figure 5. As with the salts, the activity of "20%" LDH is, in general, relatively greater at a given osmolyte concentration than is that of "100%" LDH, but the differences are much smaller. With the possible exception of alanine, the aliphatic amino acids are only marginally inhibitory, even at high (0.5 M) concentrations; the longer the chain length, the more innocuous the amino acid, so that leucine and isoleucine have little effect on activity, even at 0.5 M. Likewise, the longer of the two hydroxyamino acids, threonine, is more innocuous than serine. Thus, the more nonpolar an amino acid, the less its inhibitory effect.

The effects of taurine, betaine and TMAO are shown in Figure 6. The tri-

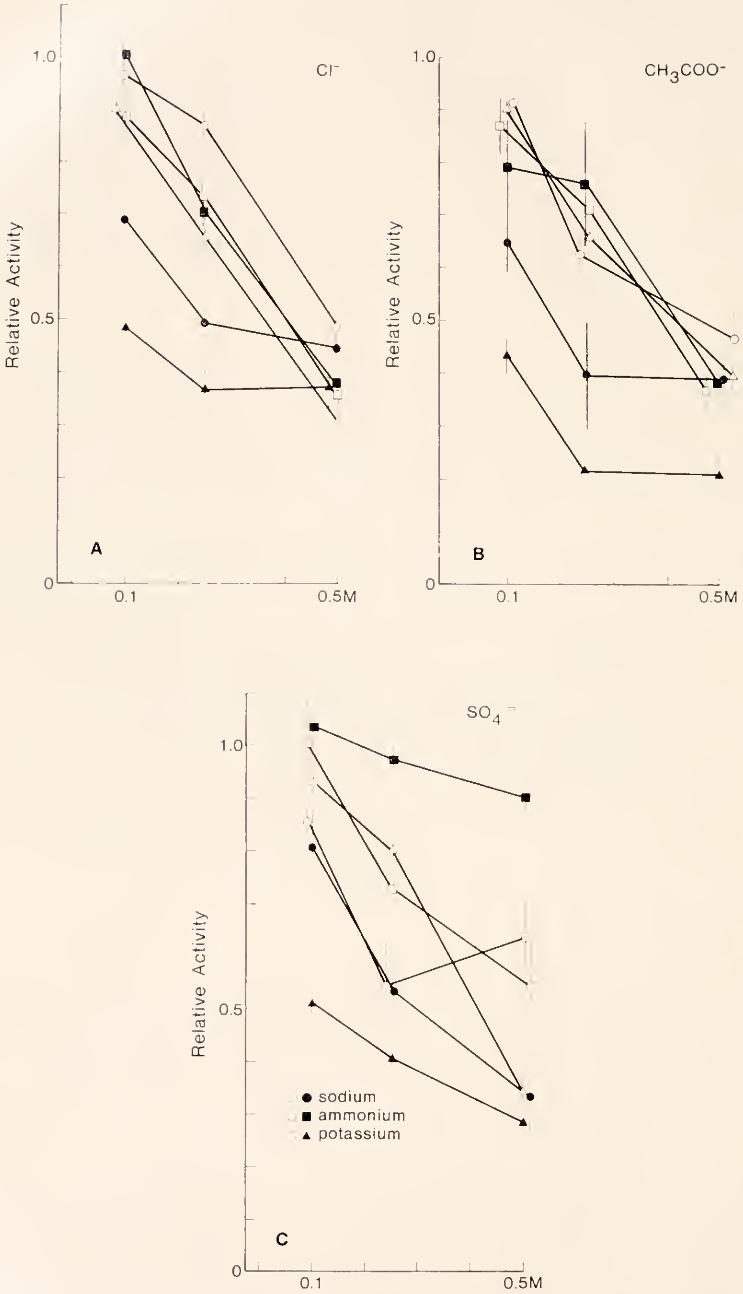

FIGURE 3. Effects of cations on LDH activity with (a) chloride (b) acetate and (c) sulfate counterions. Circles represent sodium; squares, ammonium; and triangles, potassium. Filled symbols are "100%" LDH; open symbols are "20%" LDH. Bars indicate range of two replicates.

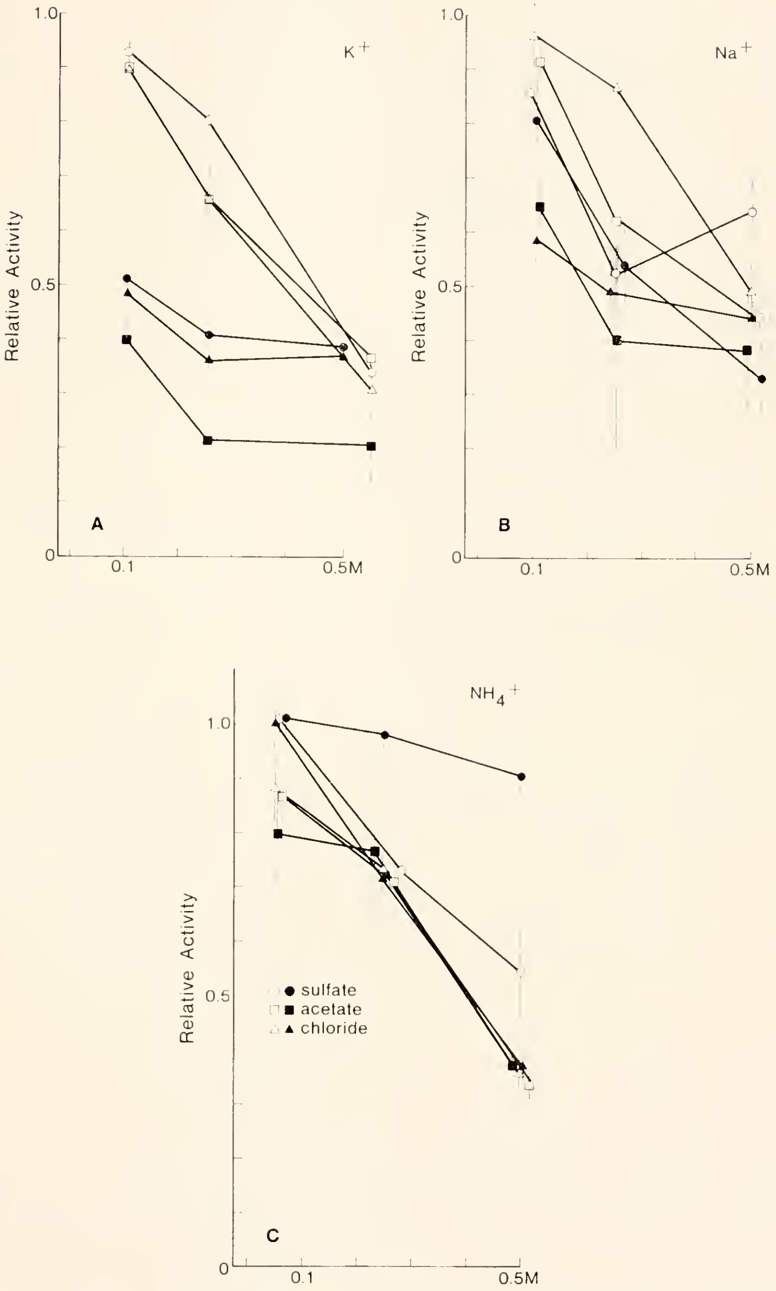

FIGURE 4. Effects of anions on LDH activity with (a) potassium (b) sodium and (c) ammonium counterions. Circles represent sulfate; squares, acetate; and triangles, chloride. Filled symbols are "100%" LDH; open symbols are "20%" LDH. Bars indicate range of two replicates.

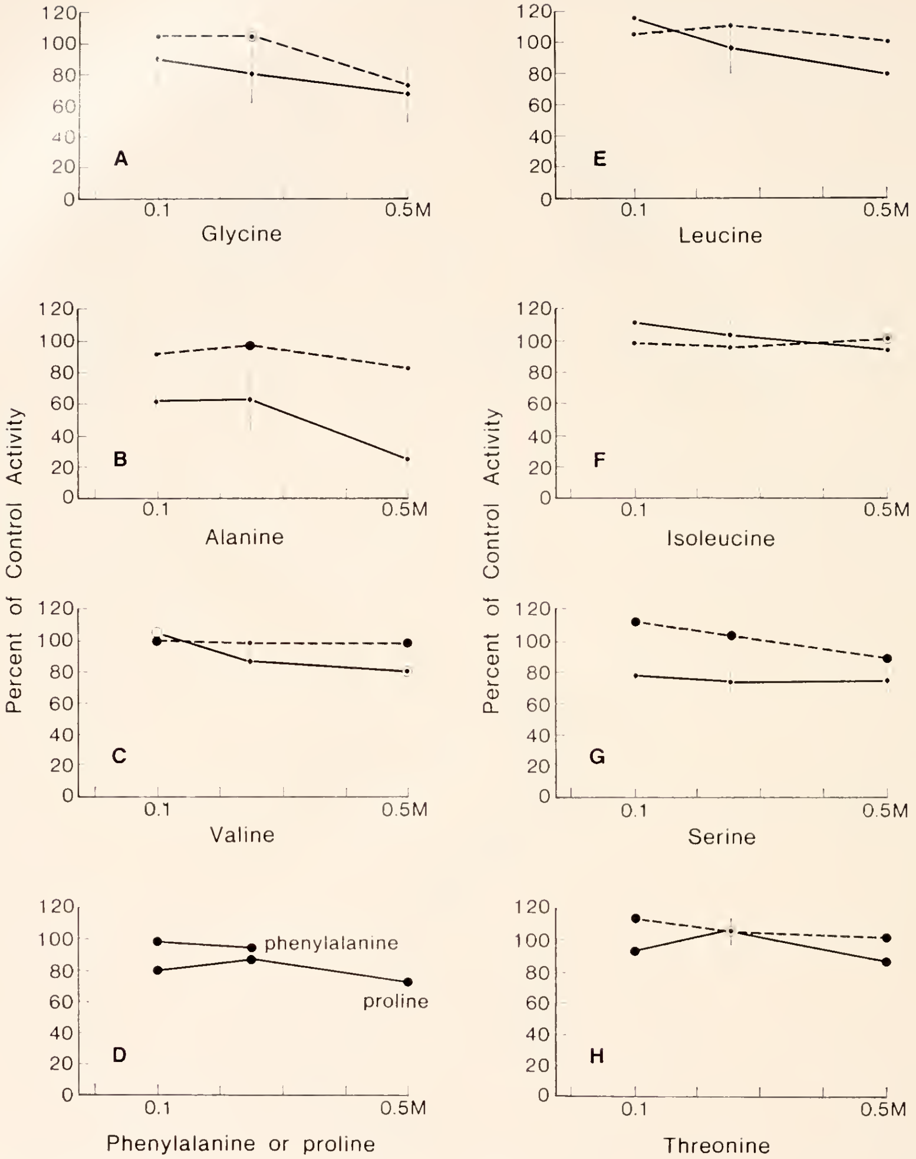

FIGURE 5. Effects of neutral amino acids on LDH activity. Solid lines are "100%" LDH; dashed lines are "20%" LDH. Symbols for replicate agreement are: circle with dot, two replicates differ by >20%; filled circle, two replicates differ by >10%; open circle, two replicates differ by <10%; dot with line, standard deviation of three replicates; dot, single determination.

substituted amines are relatively innocuous; whereas taurine, despite careful buffering, is inhibitory, especially to "100%" LDH.

When TEA or TMA was substituted for TRIS as buffer, there was generally

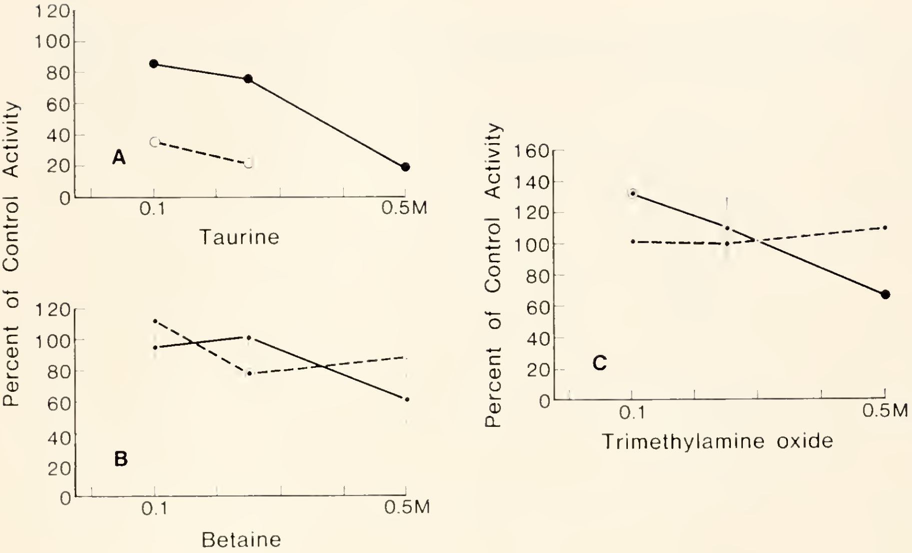

FIGURE 6. Effects of taurine, betaine and TMAO on LDH activity. Solid lines are "100%" LDH; dashed lines are "20%" LDH. Symbols for replicate agreement are the same as in Figure 5.

little difference in enzyme activity, either in the controls or in the presence of various nitrogenous organic solutes. An exception occurred in the case of taurine, however. Compared to the 0.1 M TRIS control, 0.25 M taurine plus 0.1 M TRIS gave about 70% activity; substitution of TEA for TRIS gave about 85% activity, and of TMA, about 120%. It is thus apparent that significant synergistic interactions may occur among cell solutes that, with our present knowledge, cannot be predicted from data on the effects of individual solutes alone.

Effects of solutes on apparent K_m of pyruvate

In order to evaluate the probable physiological effects of cell solutes on LDH activity, the apparent pyruvate K_m (substrate concentration where $v = V_{max}/2$) in the presence of various added solutes was measured from double reciprocal plots of $1/v$ versus $1/(S)$. The results are shown in Table I, together with values of r^2, the coefficient of determination, which expresses the goodness of fit of the regression. Given the error observed above in replicate determinations, we consider that the slopes are linear in all cases; furthermore, K_m values differing by less than a factor of two are not considered to be significantly different.

The results for "100%" and "20%" LDH are not significantly different. Glycine at 0.25 M has no significant effect on K_m, whereas KCl at 0.375 M and 0.5 M approximately triples the the pyruvate concentration required for half maximal velocity. The further addition of glycine has little effect, suggesting that intracellular glycine does not reverse the inhibitory effects of neutral salts, but merely acts as an innocuous substitute osmolyte. On the other hand, 5% bovine serum

TABLE I

Effect of selected solutes on the apparent K_m *of pyruvate for "100%" and "20%" LDH.*

Assay conditions	K_m of pyruvate moles × 10^{-4}/liter	r^2 value (coefficient of determination)
"100%" LDH		
0.1 M TRIS, pH 7.0 buffer only	3.7	0.925
0.25 M glycine	6.2	0.876
0.375 M KCl	17.1	0.929
0.5 M KCl	15.6	0.995
0.25 M glycine + 0.375 M KCl	18.0	0.860
5% BSA	5.8	0.955
0.25 M KCl + 5% BSA	6.7	0.998
"20%" LDH		
0.1 M TRIS, pH 7.0 buffer only	4.6	0.998
0.25 M glycine	5.6	0.978
0.375 M KCl	13.9	0.995
0.5 M KCl	19.9	0.975
0.25 M glycine + 0.375 M KCl	15.1	0.933

albumin (BSA) is capable of reversing the deleterious effects of 0.25 M KCl on the apparent pyruvate affinity, although alone its does not enhance substrate binding.

DISCUSSION

The main emphasis of this study has been on the effect of various intracellular solutes on the activity of LDH obtained from *Nereis succinea*. Before discussing these results, however, it is necessary to consider whether we are dealing with a single enzyme or several isozymes. Long and Kaplan (1968) found that LDH from the related polychaete, *Nereis virens*, is specific for D-lactate, in contrast to the L-specific LDH's of mammalian tissues (Kaplan, 1964). D-LDH's exist as dimers, in contrast to the tetrameric L-LDH's of mammals, and typically have molecular weights of 65,000 to 75,000 daltons (Long and Kaplan, 1973). It is thus likely that a dimeric D-LDH also occurs in *Nereis succinea*. This would permit the existence of three forms of LDH, assuming the presence of two different subunits and, indeed, three LDH bands can be detected after gel electrophoresis of *N. succinea* homogenates (Jones, 1970; Gordon Lusk, San Diego State University, unpublished data). Whether these isozymes have different catalytic properties, however, remains to be determined. Simple hyperbolic behavior with a given homogenate was observed in the present experiments, suggesting that if several isozymes are present, they are kinetically similar.

On the other hand, the data on the differential effects of osmolytes on "100%" and "20%" LDH's suggest that osmotically adaptive isozymes may be produced by *Nereis*, despite the fact that Lusk found no detectable differences in LDH isozyme patterns in homogenates from 100% and 20% SW-adapted worms. At least one species, the brook trout, is known to be capable of forming environmentally adaptive LDH isozymes, in this instance in response to acclimation to various temperatures (Hochachka and Lewis, 1971). Even though evidence for the presence of osmotically modulated LDH isozymes is not conclusive, it is interesting to speculate briefly on the possible adaptive significance of a low tissue concentration of a salt-sensitive LDH in osmoconforming worms ("100%" LDH) and a doubled tissue concentration of a salt-resistant enzyme in osmoregulating worms ("20%" LDH). Since an alternative pathway for pyruvate is reductive amination to alanine, at high osmolarity, when a large amino acid pool is maintained, low tissue levels of a potassium ion-inhibited LDH would favor alanine synthesis. Conversely, during osmoregulation when the amino acid pool is lowered (while potassium ion concentration remains little changed), an increase in tissue LDH, of a form no longer sensitive to ion inhibition, would facilitate accumulation of lactate.

Although differences in solute effects on "100%" and "20%" LDH's from *N. succinea* appear to exist, the overall responses of the enzymes to various categories of solutes are sufficiently similar that they can be considered together in the subsequent discussion. In general, neutral salts at concentrations equivalent to those found in sea water or extracellular fluids of *Nereis succinea* (Freel *et al.,* 1973) are highly inhibitory for LDH activity and tend to follow the lyotropic series. On the other hand, with the exception of taurine and possibly alanine, nitrogenous solutes appear to be largely innocuous for LDH activity. The fact that acetate ion, alanine and taurine are all far more inhibitory than predicted by our initial hypothesis may be explained by the similarity of these osmolytes to the substrate, pyruvate. Marginal competitive inhibition may be occurring, but this has not been tested. The possibility of species specificity in the activation or deactivation of LDH by various solutes must also be considered. Taurine, for example, although present in most marine invertebrates, ranges widely, from 6 to 106 μmoles per gram wet muscle tissue, even among species of a single phylum, the molluscs (Awapara, 1961; Schoffeniels and Gilles, 1972). Co-evolution of macromolecular structure with solute composition remains as a possible explanation in this instance.

The mechanism by which certain neutral salts partially inhibit enzyme activity is not yet completely clear, although the work of Somero *et al.* (1977) strongly suggests that it is not the catalytic site *per se,* but rather the catalytic conformational changes of the enzyme that are being affected. The effective salt concentrations seem too high for the ions to be acting as specific active site or allosteric site inhibitors; K_i would be in the range 0.1 to 1.0 M, depending on the salt. Thus, although complete denaturation has obviously not occurred, 0.5 M concentrations of salts such as NaCl and KCl must be modifying the conformational structure of the enzyme sufficiently to affect its rate limiting steps. The present results indicate that not only is the overall catalytic rate of the enzyme greatly affected by neutral salts, but that the apparent substrate binding affinity is also significantly reduced (Table I). Hence, an enzyme from a euryhaline polychaete whose body fluids contain high salt concentrations (Freel *et al.*, 1973) is similar to mammalian enzymes (Warren *et al.*, 1966; Warren and Cheatum, 1966) in its susceptibility to salt inactivation.

In the case of LDH, one possible effect of such salts is to cause dissociation of subunits, as demonstrated by Eichner (1973) on LDH from the lobster, *Homarus americanus.* He observed that ammonium sulfate raised to a concentration of 1.3 M caused dissociation of tetramers to dimers, with concomitant loss of activity. However, since such charge-initiated subunit dissociation is unique to this species, and requires a very high ionic strength, it is unlikely to account for the inhibitory effects of uni-univalent salts at 0.2 to 0.5 M, nor can it explain the fact that different salts have different effects at similar ionic strengths. Other, more subtle salt-induced changes in conformation appear to be affecting catalytic activity. One theory, suggested by Klotz (1965) and modified and elaborated by Lewin (1974) is that neutral salts differ in their action at the macromolecule-solvent interface, thus affecting the quantity and quality of the hydration sphere which in turn determines macromolecular conformation.

The results obtained here on a representative macromolecule, LDH, thus tend to confirm our first two hypotheses: first, that macromolecules of a marine invertebrate are indeed susceptible to functional inhibition by concentrations of those neutral salts found in its environment; and secondly, that lyotropically more favorable salts, such as ammonium sulfate, or nitrogen-containing organic solutes with comparable functional groups, such as amino acids or TMAO, are innocuous to macromolecular function. Although not all nitrogenous solutes are equally favorable toward LDH function, in general they are considerably less deleterious than the major intracellular ions, potassium, sodium and chloride. Since, according to this hypothesis, it is an osmotic equivalence rather than a molar equivalence of neutral salts that must be replaced by organic solutes, the results on several solutes were compared by plotting them as a function of both molarity and osmolarity (Fig. 7). The osmotic equivalence scale is based on the assumption of equal osmotic coefficients for all solutes. This gives a conservative comparison between salts and amino acids, since 0.5 M KCl, for example, has an osmotic coefficient of about 0.90 (Scatchard, Hamer and Wood, 1938) whereas that of 0.5 M amino acids is around 1.0 (Robertson, 1975).

So far, however, there is no proof that the third hypothesis is correct, namely that small nitrogenous organic solutes can offset the effects of functionally deleterious solutes. In the single attempt to test this, the amino acid glycine was without significant effect on the apparent K_m of pyruvate, either in the presence or absence of salts. Results with this one amino acid, however, may not reflect the potential of less polar amino acids, such as valine and isoleucine, to protect the enzyme in the presence of neutral salts. Nor do other researchers appear to have critically tested whether or not solutes capable of stabilizing macromolecular conformation, such as amino acids and ammonium sulfate, are also able to protect them against denaturation by lyotropically active ions.

Of special interest in this regard is the observation that 5% BSA is capable of suppressing the increase in apparent K_m of pyruvate induced by moderate KCl concentrations (Table I). Although intracellular chloride ion concentrations in *Nereis succinea* are low (33 mM in 100% SW-adapted worms) the potassium ion concentration is about 234 mM (Freel *et al.,* 1973); and it is this ion which is most deleterious to "100%" LDH, 0.25 M K^+ resulting in 60 to 80% reduction in activity regardless of the counterion present (Figure 3). Although the intracellular soluble protein levels in *Nereis* have not been measured, recent studies on the total intracellular dry weight components of muscle from another marine invertebrate, the giant barnacle *Balanus nubilus,* indicate that soluble macromolecules capable of separation by ultracentrifugation are present at a concentration of some 7 g per 100 g cell water. More than half of this material is protein (M. Clark, unpublished observations). If comparable amounts of soluble intracellular protein are present

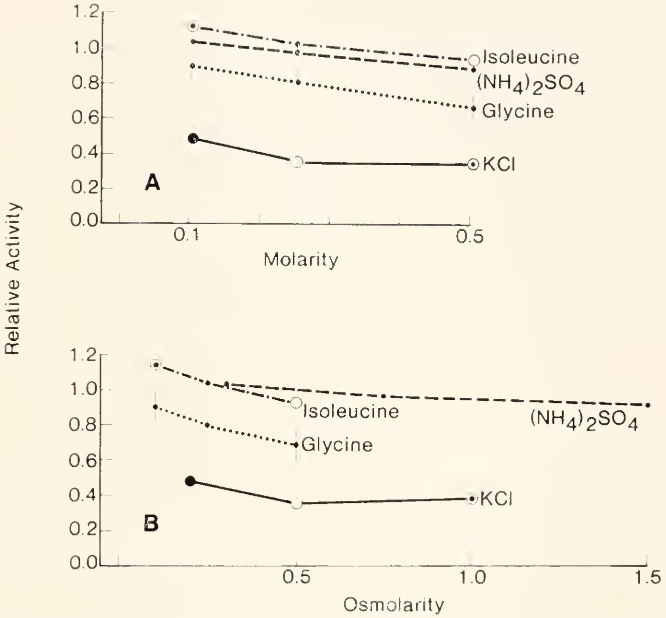

FIGURE 7. Effects of various solutes on the relative activity of "100%" LDH expressed in terms of (a) molarity and (b) osmolarity. Symbols for replicate agreement are the same as in Figure 5.

in *Nereis*, then a 5% BSA solution is a reasonable approximation to the normal intracellular state.

These experiments suggest that small nitrogenous solutes act as relatively innocuous substitute osmolytes for deleterious ions when water is limiting in a cell and that the soluble cytoplasmic proteins play a role in protecting other macromolecules against the functionally disruptive effects of the ions that remain. It is also possible that less polar nitrogenous osmolytes may act to offset the deleterious effects of ions or more polar organic solutes, as suggested both by the fact that less polar amino acids are more innocuous to enzyme function, and by the remarkable improvement in LDH activity observed on substituting less polar TMA for more polar TRIS in the presence of the highly polar solute, taurine. Nonpolar side groups may act at the macromolecular-water interface to increase water activity and, hence, decrease bound water at the surface. There would therefore be a decrease in activation energy for conformational change during catalysis (Lewin, 1974).

Given the fact that even 0.1 M K^+ reduced "100%" LDH activity by 50% (although its effect on "20%" LDH is much less), one may consider whether intracellular potassium ion in freshwater or terrestrial species may not also be potentially deleterious to the function of their macromolecules. For example, the intracellular potassium ion concentration in frog (*Rana pipiens*) muscle is 141 mM (Lee and Armstrong, 1974), and in various rat muscles it ranges from 142 to 178 mM (Drahota, 1961). Either the enzymes and other macromolecules of these species are, like "20%" LDH from *Nereis*, relatively unaffected by such potassium ion concentrations, as appears to be the case for halibut muscle LDH (Somero *et al.*, 1977), or they are protected by soluble protein or other solutes within the cell, or they are functioning at sub-optimal levels. The general applicability of the results observed here, both in terms of other species and other macromolecules, remains to be investigated. These experiments indicate the potential importance of the entire complement of intracellular solutes in determining the physiological level of activity of cell macromolecules.

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SUMMARY

1. In searching for an explanation for the high intracellular concentrations of nitrogenous organic osmolytes found in all marine invertebrates, the similarity of their functional groups to those neutral salts known to stabilize native macromolecular conformations was noted. This led to the hypothesis that such osmolytes are innocuous and substitute for more deleterious salts at high osmotic pressures. The hypothesis was tested on a representative macromolecule, the enzyme lactate dehydrogenase (LDH), from the euryhaline polychaete, *Nereis succinea*.

2. The activity of LDH from worms adapted to a range of salinities was measured at several pH values and in the presence of various concentrations of neutral salts and of low molecular weight nitrogenous solutes characteristically found intracellularly in marine and euryhaline invertebrates.

3. Differences in LDH activity in homogenates from worms adapted to osmoconforming (100%–35% SW) and osmoregulating (20% SW) salinities were observed. Enzyme activity from osmoregulating worms ("20%" LDH) occurred at nearly twice the concentration in tissue, and was less inhibited by neutral salts than was "100%" LDH. These differences may reflect the synthesis of a new isozyme during osmoregulatory adaptation, designed to divert pyruvate from entering the free amino acid pool by more readily converting it to lactate.

4. Low molecular weight nitrogenous solutes are generally far less inhibiting to LDH activity than are intracellular ions at similar osmolarities. The more nonpolar the compound added, the more innocuous its effect on the enzyme. This may be due either to suppression of charge-initiated dissociation of subunits, or the nonpolar groups may stabilize the macromolecule-solvent interface.

5. Although glycine is unable to protect LDH from inhibition by neutral salts, 5% bovine serum albumin is effective in this respect. This suggests that soluble cytoplasmic protein and perhaps less polar nitrogenous solutes as well play an important role in protecting macromolecules from functional inactivation by ions normally occurring in the cell.

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THE BRANCHIAL CHAMBER IN TERRESTRIAL CRABS: A COMPARATIVE STUDY

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Adaptive responses to different environments can be functional, structural, behavioral or a combination of these. The exposure to different environments can be due to changes in environmental factors or due to the invasion of new habitats by a progressive migration of organisms. Invasion of the land has required a number of such adaptations in amphibious crabs (Bliss, 1968), foremost among these being adaptations connected with respiratory mechanisms. Comparisons between aquatic and terrestrial crabs have shown that the latter have fewer gills (Pearse, 1929, 1950) and reduced gill surface per unit weight (Gray, 1957; Bliss, 1963). Also, the gills of terrestrial crabs have become highly sclerotized on the edges, presumably to keep them erect and functional in air (Harms, 1932). A connection has often been mentioned between the globose carapace of Gecarcinidae, Potamonidae, Pseudothelphusidae and Trichodactylidae and their ability for aerial respiration.

It has been suggested that, during the evolution towards a terrestrial habitat, the reduction of gills and gill area is compensated by an increasingly more important role in respiration played by the thin epithelial membrane that lines the branchial chambers (Carter, 1931; Edney, 1960). The lining of the gill chamber has been referred to by many authors as the "lung" of land crabs. Indeed, some indications of this "lung" exist in the vascularized epithelium of the branchial lining in *Uca* (Jobert, 1876) and *Cardisoma* and in the tufts arising from the branchial lining in species of *Ocypode, Coenobita* and *Birgus* (Borradaile, 1903; Harms, 1932).

Of the 4,500 species of brachyuran crabs so far known, many which live in intertidal zones can withstand various degrees of exposure to air. However, true amphibious life is restricted to a few terrestrial and freshwater forms, most of them belonging to the families Grapsidae, Gercarcinidae, Ocypodidae, Potamonidae, Trychodactylidae, Pseudothelphusidae, and Myctyridae (Bliss, 1968). In the present study, evidence is presented on the respiratory function of the lining of the branchial chamber in several semiterrestrial brachyuran species from marine, estuarine, and freshwater habitats.

MATERIALS AND METHODS

In the course of the present study, seven brachyuran species belonging to four families were used for estimates of branchial chamber volume, observations of the chamber's shape, and examination of the structure of the lining which covers the chamber. The sites of collection varied from the supralittoral zone of sandy beaches to the mountains of tropical forest and the llanos (savanna-like forest).

Material examined

Trychodactylidae: *Valdivia venezuelensis* Rathbun, *Dilocarcinus dentatus* (Randall). Type of habitat: savanna-like areas (llanos) in large rivers, ditches and ponds, either on the bottom or in deep holes in the banks of the rivers. These crabs are able to stand long periods of time out of the water (Holthuis, 1959; Valente, 1948). Site of collection: at rivers in the Venezuelan llanos (Calabozo, State of Guárico) at 150 m above sea level.

Pseudothelphusidae: *Eudaniella iturbei* (Rathbun), *Microthelphusa simoni* (Rathbun). Type of habitat: small mountain creeks and streams where they dig shallow burrows under large stones that are surrounded by water; these burrows might be extended 30 cm deep or more under the stream bed. Species of this group can live long periods of time out of the water and survive when small streams dry up during the dry season (Holthuis, 1959). Site of collection: *E. Iturbei* at small mountain creeks at 1,300 m above sea level in the Venezuelan coastal range (State of Miranda); *M. Simoni,* at same area, but only 40 m above sea level.

Gecarcinidae: *Gecarcinus lateralis* (Fréminville). Type of habitat: this species digs its burrows in dry sand high up on the beaches and dry sparse grassland where ground water is not close by and the only moisture available comes from infrequent rain showers or dew (Bliss, 1963). Site of collection: supralittoral zone of sandy beaches at the central coast of Venezuela (State of Miranda).

Cardisoma guanhumi Latreille. Type of habitat: this species inhabits low-lying areas up to 8 km away from the sea. This crab is commonly found in mangrove swamps, where it digs down to the ground water (Herreid and Gifford, 1963). This species is capable of surviving long periods of submersion in water (Gifford, 1962). Ovigerous females of *G. lateralis* and *C. guanhumi* usually migrate into the sea for spawning. Site of collection: mangrove swamps at the central coast of Venezuela (State of Miranda).

Ocypodidae: *Ocypode quandrata* Fabricius. Type of habitat: this crab is commonly found in the supralittoral zone of sandy beaches where it digs deep holes distinctively located from just above high tide mark to some 500 m into the dunes (Bliss, 1968). Adult specimens of this species are nocturnally active, but juveniles may be seen out of their burrows during the day (Hughes, 1966). Site of collection: supralittoral zone of sandy beaches, coast of Venezuela (State of Miranda).

The volume of the branchial chamber was measured in at least 32 specimens of each species (except in *Eudaniella iturbei*, where only 14 specimens were available); and, at the same time, body weight, carapace width, and carapace length were recorded. The specimens were killed by immersion in 10% formalin and weighed after removal of surplus water from the body and gill chamber with a paper towel and removal of the pereiopods. The pereiopods were amputated in order to avoid possible variability in weight due to total loss or regeneration of some limbs prior to collection. To determine the volume of the branchial chamber, melted paraffin was perfused through the efferent channels, completely filling the chamber. The cast thus obtained showed the shape of the chamber, and its volume was determined from the weight and density of the paraffin cast. Since values for both chambers were obtained, a mean value was taken for each specimen.

The general morphology of the branchial chamber of several Antillian and Central and South American species of Pseudothelphusidae was studied. *Ucides cordatus*, a gecarcinid, was also cursorily examined. The lining of the branchial chamber in all species that were studied was routinely examined.

In several pseudothelphusid species, a new respiratory structure was discovered (see results). In order to determine the kind of relationship between the size of the animal (expressed as carapace breadth) and the morphological characteristics of the respiratory structure, ten specimens of *Eudaniella iturbei,* which spanned the size range of the species, were selected. Measurements were made of the diameter of perforations found in the structure, its thickness, and the external area of the respiratory region; finally, the total number of perforations on the structure were estimated. Similar measurements were made on the other pseudothelphusid species (Table I).

To determine the localization of respiratory sites, a technique was used similar to that of Bertolini (1933, 1934) for holothurians, and Edney and Spencer (1955) for isopods. A solution of methylene blue (1.5 mg/ml, approximately) was prepared in boiled distilled water and then reduced by addition of a saturated solution of hydroquinone and sodium sulfite, hydroquinone alone, or sodium sulfite alone, until all blue color disappeared. From 1 to 5 μl of this reduced solution were injected every four hours into living crabs through the branchial veins, through the coxopodite of each pereiopod, or by direct puncture to the heart. Reduced methylene blue recovers its original color by oxidation and stains the tissues dark blue or black where oxygen uptake occurs. Crabs were sacrificed at different times after injection and the entire branchial chambers were inspected. In order to accelerate the process, some crabs were maintained in an atmosphere of nitrogen, and nitrogen was also forced into the gill chamber through the efferent channels. The animals were kept in this condition from 5 to 10 minutes, after which the heart was injected with reduced methylene blue. Immediately after injection, oxygen was forced into the branchial chamber, and the animals were maintained in an atmosphere of oxygen for not less than 15 minutes; then they were sacrificed.

Another set of specimens, not less than five per species, was used for histological study. Segments of the lining or the whole branchial chamber were fixed in Helly-Zenker solution (except for one instance, in which trichloroacetic acid was used) and stained with hematoxylin-eosin. Staining tests for the identification of mucopolysaccharides were carried out by the Periodic-Acid-Schiff (PAS) reaction, PAS inhibited by dimedone, and PAS inhibited by ptyalin (Pearse, 1960).

RESULTS

General morphology of the branchial chamber

The paraffin casts gave a three-dimensional view of the branchial chambers, as seen from the outside. In each case a dorsal, a frontal, and a lateral view of the left chamber is given (Figs. 1 and 2). The frontal portion of the crab corresponds to the upper part of the drawing in dorsal view and to the right-hand side of the drawing in lateral view. In the case of the frontal view, the left-hand side of the drawing corresponds to the lateral portion of the crab. In each view, the peripheral line of the drawing gives an approximate idea of the cross section of the chamber through each corresponding plane. The depressions shown on the surface of the drawing of the casts correspond to internal expansions and projections of the chamber lining.

Both *Cardisoma guanhumi* and *Gecarcinus lateralis* have a globular chamber, very simple in outline and without internal projections (Fig. 1, a to f). The gill chamber of *Ucides cordatus* presents the same general form; but at its lower part, just over the gills, the lining forms a fold that practically separates the gills from the rest of the chamber. In contrast to these species, *Ocypode quadrata* has a

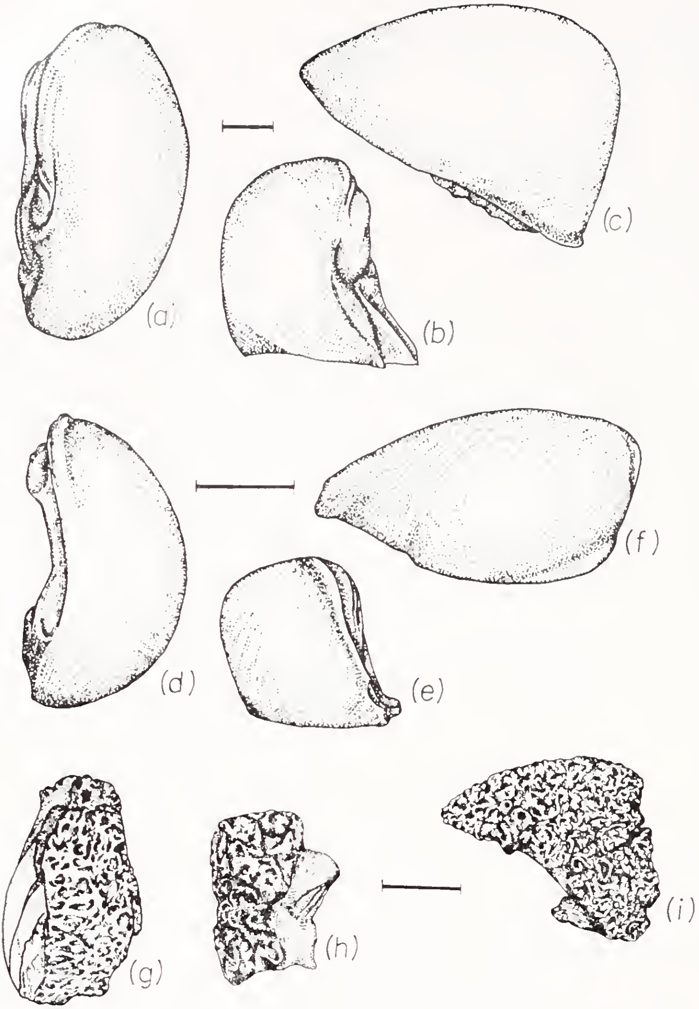

Figure 1. Shape of the lefthand branchial chamber, according to the paraffin casts obtained from different species. *Cardisoma guanhumi* Latreille: a) dorsal view; b) frontal view; c) lateral view. *Gecardinus lateralis* (Fréminville): d) dorsal view; e) frontal view; f) lateral view. *Ocypode quadrata* (Fabricius): g) dorsal view; h) frontal view; i) lateral view. Casts for drawings were selected among those obtained from the largest specimens collected. Scale bars represent 1 cm.

pyramidal chamber (Fig. 1, g to i) with very conspicuous digitations and projections all over the upper, lateral, and anterior sides of the lining. In this case, a fold similar to that shown by *U. cordatus* is also present.

The chamber of *Dilocarcinus dentatus* is somewhat similar in shape to that of *O. quadrata,* but its wall is devoid of any kind of projections; however, several folds can be detected in the floor of the chamber (Fig. 2, a to c). Neither projections nor protuberances were observed in the lining of the gill chamber of *Valdivia venezuelensis*. In this case, the chamber was found to be similar in shape to that of *D. dentatus*. The few casts obtained from specimens of *V. venezuelensis* showed the frontal and posterointernal portions reduced by the ripe gonads.

In *Eudaniella iturbei* the branchial chamber is very much reduced in the posterior portion, the chamber being restricted to a globular cavity in the anterior half. In a mature female, the dorsal plane might be reduced anteriorly by development of the gonads along the frontal area of the specimen (Fig. 2, d to f). This general description of the branchial chamber of *E. iturbei* is also valid for *Microthelphusa simoni*.

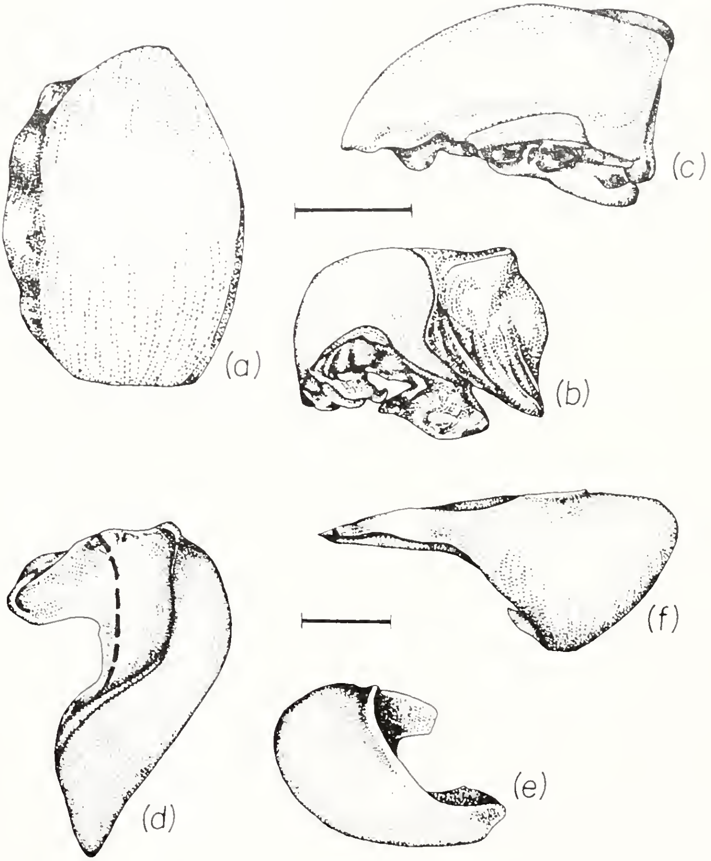

FIGURE 2. Shape of the lefthand branchial chamber, according to the paraffin casts obtained from different species. *Dilocarcinus dentatus* (Randall): a) dorsal view; b) frontal view; c) lateral view. *Eudaniella iturbei* (Rathbun): d) casts show the effect of gonad expansion into the lumen of the branchial chamber; dashed line indicates the normal outline of the chamber in the dorsal view; e) frontal view; f) lateral view. Casts for drawing were selected from those obtained from the largest specimens collected. Scale bars represent 1 cm.

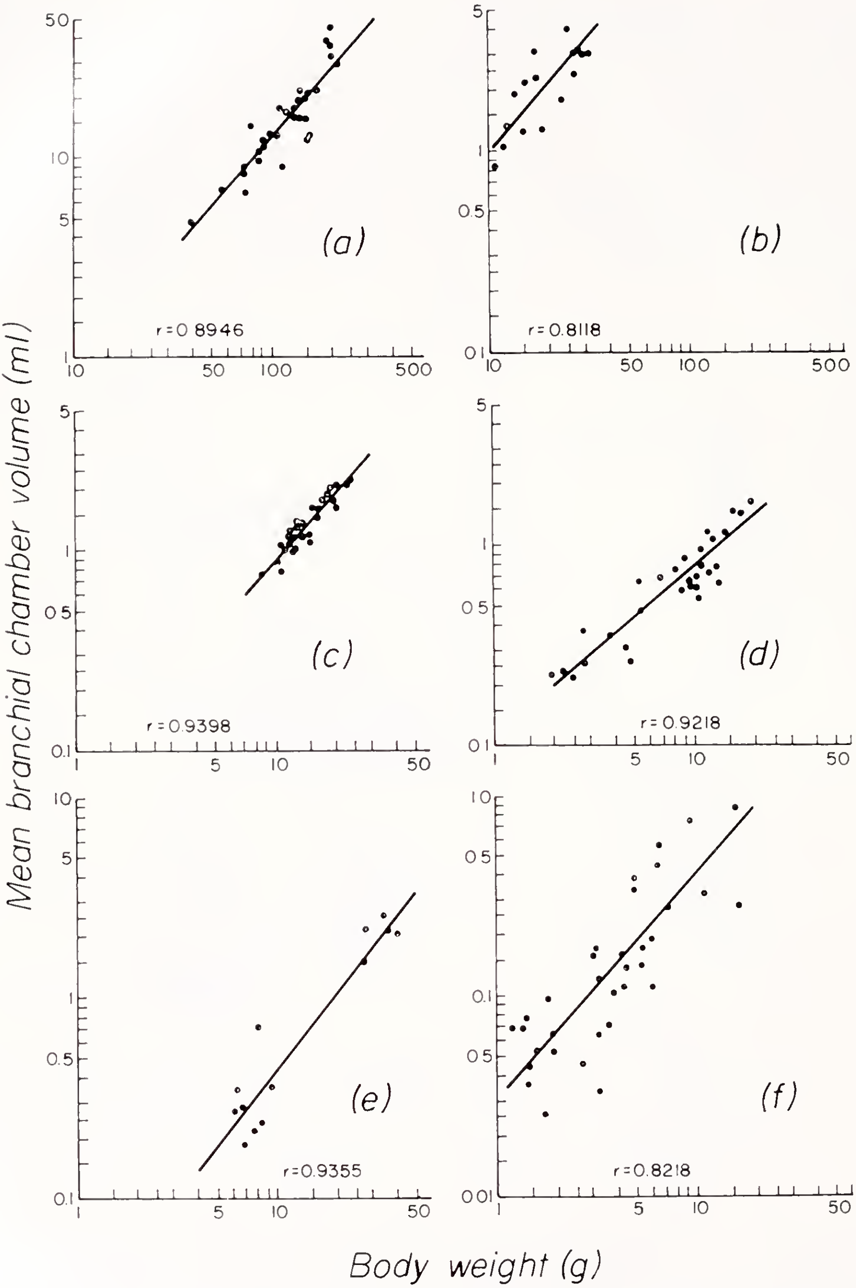

FIGURE 3. Data points and regression lines expressing the relationship between weight of the crab's body without pereiopods and the mean volume of its branchial chamber: a) *Cardisoma guanhumi*, $y = 0.0614x^{1.158}$; b) *Gecarcinus lateralis*, $y = 0.0786x^{1.145}$; c) *Dilocarcinus dentatus*, $y = 0.0636x^{1.135}$; d) *Ocypode quadrata*, $y = 0.1062x^{0.862}$; e) *Eudaniella iturbei*, $y = 0.0235x^{1.272}$;

Relative growth of the branchial chamber

Regression lines were obtained by relating the weight of the crab's body without pereiopods (x) and the branchial chamber volume (y) (Fig. 3). For all these cases, a linear equation expresses the relationship between the logarithms of the two variables, with a correlation coefficient greater than 0.81 ($P < 0.01$); thus, an allometric growth of the branchial chamber was assumed for all the species considered in this section. Bartlett's method was used to determine the relationship between logarithms (Simpson, Roe and Lewontin, 1960). Comparisons were made among the six species to test for differences between each pair of values of slopes and also for the values of y, when $x = 1$. These tests revealed that the slopes were not significantly different ($P < 0.05$). However, comparisons made among the points of intersection suggested that there were three categories of branchial chamber development. A group with a relatively large branchial chamber was represented by *C. guanhumi, G. lateralis,* and the river crab *D. dentatus.* A group with a relatively small branchial chamber was formed by the mountain freshwater crabs, *E. iturbei* and *Microthelphusa simoni;* and an intermediate branchial chamber group was represented by *O. quadrata.*

The regression lines obtained for the species forming each of the extreme groups did not show any significant differences ($P < 0.05$ with respect to slope and intersection on the y-axis. Each group or category proved to differ significantly ($P < 0.05$ from the other two only in the y-intersection.

The perforated area or "lung"

All the species of Pseudothelphusidae examined show a distinctive perforated area located on the ceiling of the branchial chamber wall over both anteroexternal angles of the body. The general illustration given in Figure 4 is for *Eudaniella iturbei,* but similar structures were observed in all pseudothelphusids from Venezuela, as well as in all of the specimens from Central and South America and the Antilles. *Potamocarcinus mociñoi,* a Mexican cave dwelling pseudothelphusid, was the only species examined that did not show this characteristic perforated area. The lining of the branchial chamber was devoid of any kind of specialized structure.

The perforated area is formed by small holes closely placed in an oval distribution. The area of the perforated zone, the density of perforations, the mean diameter of the channels, and the thickness of the structure are related to the size of the animals and to the species considered. Table 1 shows the values obtained for several parameters measured on the perforated area of several species of Pseudothelphusidae.

Unfortunately, there were not enough available specimens of some species to confidently establish interspecific comparisons. However, the general trend of variation of the characters measured was obtained for one species. Figure 5 shows the data points and the respective regression lines that establish the relationships between the carapace breadth of *Eudaniella iturbei* and the measured characteristics of the perforated area found in the lining of its branchial chamber. For this species,

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and f) *Microthelphusa simoni,* $y = 0.0311x^{1.140}$. The correlation coefficient for each of the regression lines is given.

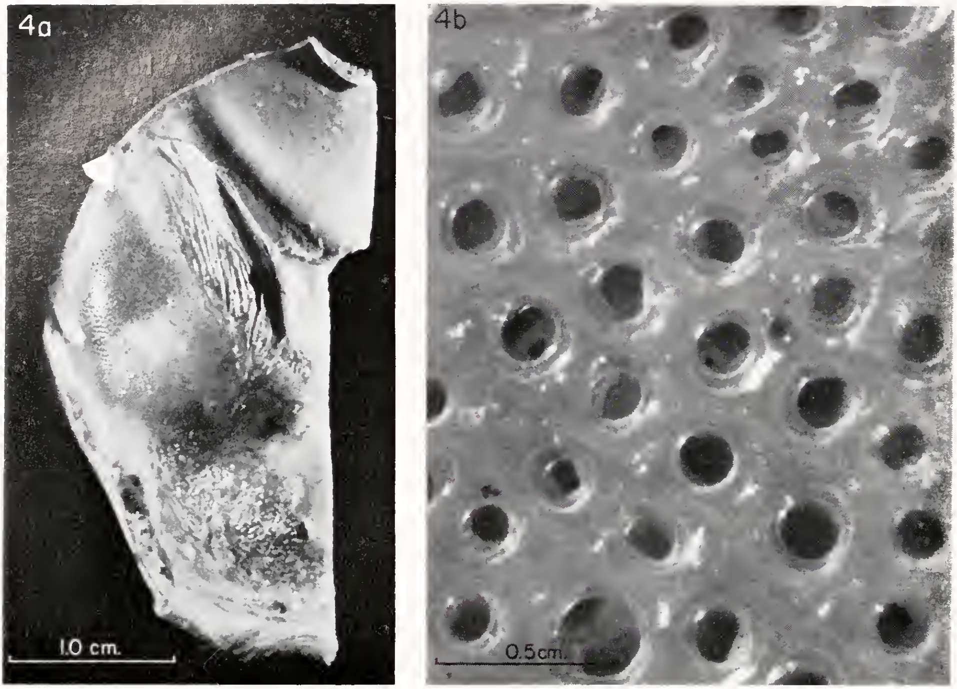


FIGURE 4. *Eudaniella iturbei* (Rathbun): a) ceiling of the branchial chamber; and b) detail of the exterior surface of the perforated area found in the ceiling (anterolateral angle) of the branchial chamber.

the data revealed linear relationships between the mean diameter of the holes and the carapace breadth (x) (Fig. 5a), as well as the thickness of the structure and the carapace breadth (Fig. 5b). A negative power equation ($y = bx^{-c}$) was determined for the regression line obtained when comparing the carapace breadth with the estimated mean number of holes per mm^2 (Fig. 5c). In the case of the external area of the perforated region (y), a power equation appeared to be the best fit (Fig. 5d). A correlation coefficient above 0.94 was found for all these cases, being significant at 0.01 level. Using the estimated density of perforations and the total area of the structure considered, an estimate was obtained for the total number of perforations. There was no significant relationship ($P > 0.05$) between the size of the animal and the total estimated number of perforations.

Sites of oxygen uptake

Assays made with reduced methylene blue gave similar results, regardless of the reduction agent used. In all cases the branchial lamellae of the specimens were stained dark-blue or black within five to 24 hours. The process was accelerated (10–15 min) when the crabs were kept for a few minutes in air deprived of oxygen, injected with methylene blue, and subsequently transferred to an atmosphere rich in O_2. Besides the staining of the lamellae, in *Cardisoma guanhumi* and *Gecarcinus lateralis*, the entire lining of the branchial chamber was also stained in addition to

TABLE I

Dimensions of the "lung" found in several species of Pseudothelphusidae.

Species and localities of collection	Specimen number	Carapace breadth (mm)	Mean diameter perforations (mm)	Mean number perf./mm²	Mean thickness (mm)
Pseudothelphusa dugesi belliana Rathbun, El Serpentin, Michoacan State, Mexico	1	24.3	0.0513	29.15	1.0064
	2	36.0	0.1071	20.36	0.7850
	3	41.7	0.1087	13.04	1.5097
Pseudothelphusa dugesi dugesi Rathbun, Chignahuapan, Puebla State, Mexico	1	25.0	0.0701	32.87	—
	2	38.6	0.0796	23.75	1.1474
Pseudothelphusa jouyi Rathbun Camecuaro, Michoacan State, Mexico	1	30.3	0.0513	12.90	0.2738
Perforations (ϕ = 0.05 mm) were observed over the entire chamber's lining. An inconspicuous "lung" was found from which measurements were made.					
Pseudothelphusa dilatata Rathbun, Las Estacas, Morelos State, Mexico	1	44.0	0.0993	29.47	1.5097
	2	45.5	0.1047	25.21	1.0266
A well formed "lung" was observed, accompanied by perforations (ϕ = 0.0797) scattered (9.71 mm²) over the chamber lining.					
Neopseudothelphusa fossor (Rathbun) Chupsa, Miranda State, Venezuela	1	29.7	0.0900	33.88	0.8639
	2	26.8	0.0653	32.24	0.5667
Eudaniella garmani (Rathbun) Rio Nuevo, Sucre State, Venezuela	1	82.9	0.2317	8.12	2.4000
Eudaniella ranchograndensis (Rodríguez) Limón River, Miranda State, Venezuela	1	41.0	0.0740	37.58	0.8166
Potamocarcinus maxillipes (Rathbun) Teapa, Tabasco State, Mexico	1	68.1	0.1248	8.77	1.2882
Potamocarcinus mociñoi (Rioja) Cuevas del Tio Ticho, Comitán, Chiapas State, Mexico	Three specimens were examined, none of which presented any conspicuous structure that resembled the "lung" commonly found in the other species. The lining was smooth and thin without any folds or protuberances.				
Potamorcarcinus armatus Milne Edwards, Near Granada, Gran Lago, Nicaragua	1	67.1	0.0860	11.32	1.3666
Epilobocera sinuatifrons (A. Milne Edwards) El Yunque, Luquillo and near Mayaguez, Puerto Rico	1	18.6	0.0316	245.15	0.2233
	2	73.5	0.1232	11.61	1.5040
	3	80.8	0.1433	9.05	1.5733
	4	97.1	0.1548	7.45	2.9330
Guinotia dentata (Latreille) Syndicate Road, Dominica	1	62.0	0.1469	13.14	1.2220

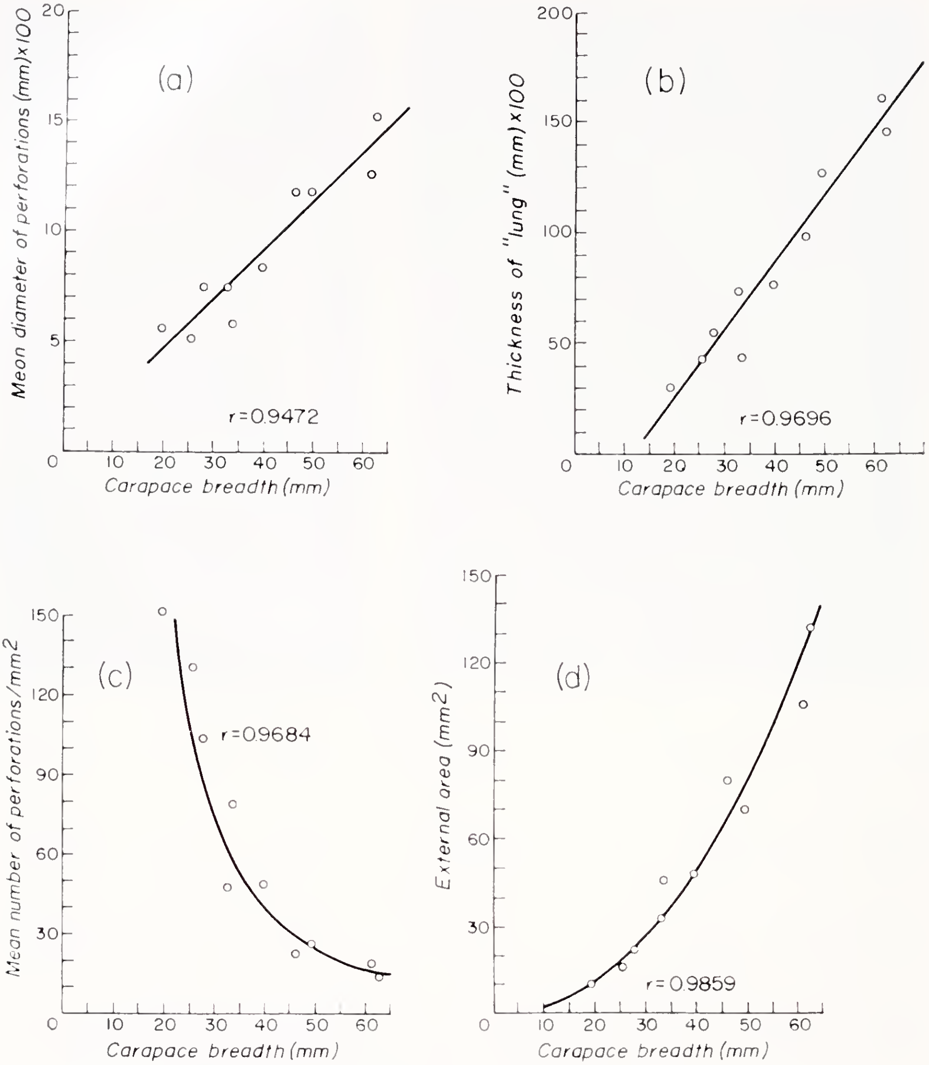


FIGURE 5. *Eudaniella iturbei* (Rathbun). Data points and regression lines express the relationships between carapace breadth (taken as expression of size of the animal) and several characters measured from the "lung" located in the lining of the branchial chamber: a) diameter of the perforation, $y = 0.2043 + 0.2236x$; b) thickness of the structure, $y = -34.632 + 3.029x$; c) number of perforations per mm^2, $y = 92276.57x^{-2.0962}$; and d) total external area estimated for the perforated structure, $y = 0.0186x^{-2.1433}$. The correlation coefficient for each of the regression lines is given.

the branchial lamellae. In the pseudothelphusids, *Eudaniella iturbei* and *Neopseudothelphusa fossor*, only the branchial lamellae and the perforated area of the chamber's lining were stained; the chamber's lining itself was not stained. Similarly, in *Ocypode quadrata* only the lining's folds were stained in addition to the gills.

Structure of the branchial chamber wall

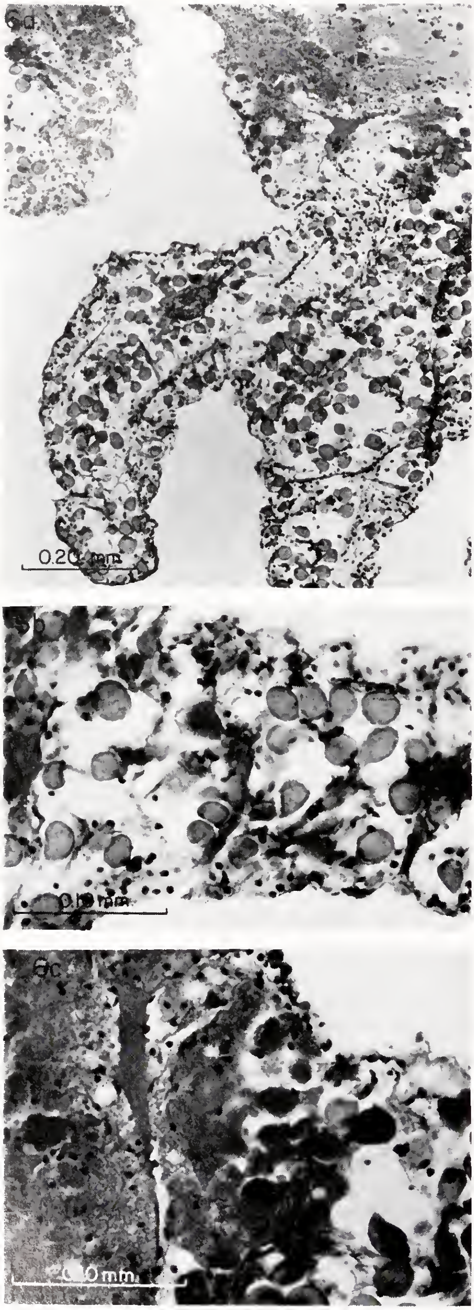
In *Ocypode quadrata*, as in other species studied, a section normal to the carapace surface (Fig. 6, a to c) showed a thin external layer of connective tissue, with groups of amber-colored melanin granules, corresponding to the chromatophores. The middle layer was a spongy tissue, with numerous blood vessels branching off towards the lumen of the chamber, and the internal layer was an epithelium composed of a simple cell layer. In the digitations and projections, thin blood vessels were particularly abundant, and numerous nonstriated muscle fibers were observed to reach almost to the internal epithelium (Fig. 6c). There were numerous birefringent spheres throughout the wall and particularly in the digitations, usually eosinophilic but sometimes basophilic in character. Such spheres possessed a PAS-positive substance, and their content sometimes showed a granular appearance. In these instances, the contour became less distinct, and spheres seemed to empty into the stroma of the tissue (Fig. 6c).

In *Cardisoma guanhumi* the layer of spongy tissue immediately below the chromatophore layer (Fig. 7a) had groups of cells organized in a pattern similar to a glandular tissue (Fig. 7, b to e). There appeared to be different stages of organization, either as groups of closely packed cells radiating from the center, or disposed as a thin-walled glandular structure whose lumen was filled with granular eosinophile substance (Fig. 7, b to e). For the rest, the branchial chamber wall had large blood vessels, and some nonstriated muscular fibers arranged tangentially and transversely wrapped in sheets of connective tissue. The internal layer was a thin epithelium (Fig. 7f). The structure of the wall in *Gecarcinus lateralis* (Fig. 8, a to d) was similar to that of *Cardisoma guanhumi*, but the gland-like structures were not observed.

The three layer structure was also present in the Pseudothelphusidae (Fig. 9a, b). Large birefringent PAS-positive spheres and a few fibers of nonstriated muscle were observed in the middle layer of the peripheral area of the perforated region on the lining or "lung" (Fig. 9b). The "lung" presented numerous channels or perforations, more or less straight in longitudinal section (Fig. 9a), but of irregular shape in cross section (Fig. 9d). The walls of these channels possessed folds (Fig. 9a, c) that sometimes gave rise to anastomosis between the channels.

The tissue among the channels was the characteristic spongy tissue of the middle layer, with numerous blood vessels and sinuses (Fig. 9, c to e). The internal side of the channel was lined with the usual one-celled epithelium found in the rest of the branchial chamber walls examined.

Valdivia venezuelensis and *Dilocarcinus dentatus* showed the basic structure of other species (Figs. 10 and 11), with an external layer with melanin granules, a middle layer of spongy tissue with numerous blood vessels and sinuses, and an internal one-celled epithelium. In the middle layer there were conspicuous birefringent PAS-positive spheres (Figs. 10 and 11) and fibers of nonstriated muscle covered by a sheet of connective fibers, arranged in a longitudinal and transverse pattern. In *Valdivia venezuelensis*, the nonstriated fibers crossed the wall from the external to the internal sides (Fig. 10d). In addition, this species possessed striated fibers, more numerous on the lateral sides of the chamber. Groups of cells with large nuclei were observed at the insertion point of these striated muscular fibers (Fig. 10b, c).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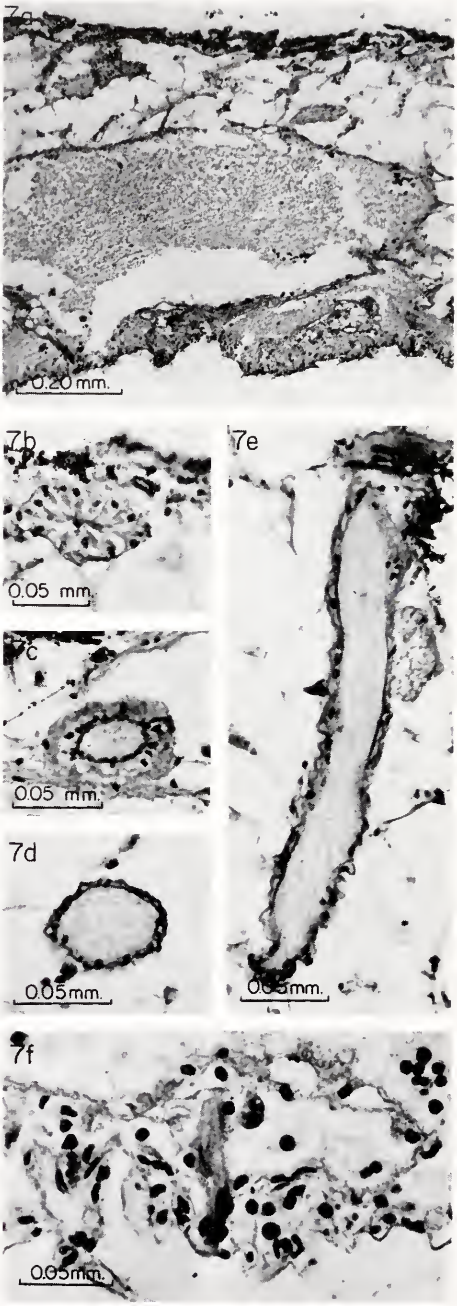
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FIGURE 6. *Ocypode quadrata* (Fabricius): a) low power view of a transverse section of the branchial chamber lining; b) view at higher magnification of the middle area of the transverse section of a fold; c) view of the tissue showing the granular aspect of some birefringent

Discussion

Certain morphological characteristics of land crabs have been seen as expressions of adaptation to their habitats. Generally, the more terrestrial forms have fewer gills and a reduced gill area when compared to more aquatic groups, regardless of their phylogenetic relationship (Gray, 1957; Veerannan, 1974). In general, land crabs have a large branchial chamber which is very vascularized and may contain folds (Edney, 1960). These characteristics have been used by several authors as indications of a "lung" in amphibious crabs, (Jobert, 1876; Borradaile, 1903; Harms, 1932; Carter, 1931; Zoond and Charles, 1931).

The suggested respiratory function of the chamber's lining in amphibious crabs was confirmed by the oxidation of methylene blue for all the species tested. Furthermore, the staining was restricted to the gill lamellae and particular areas of the chamber's lining, *i.e.,* the perforated area of *Eudaniella iturbei* and *Microthelphusa simoni,* and the folds of the chamber's lining in *Ocypode quadrata.* Although the methylene blue test was carried out in only two pseudothelphusid species, we believe the results can be extrapolated to the other pseudothelphusids examined, with the exception of *Potamocarcinus mociñoi.* This was the only species examined which did not show the perforated area.

The lamellae of the gills of terrestrial crabs appear to be strengthened with chitinous ridges, which presumably keep them erect and functional in air (Harms, 1932). The air flow inside the branchial chamber apparently occurs by the beatings of the scaphognatite (Cameron, 1975), which provides the oxygen taken by diffusion through the existing water film on the lamellae (Valente, 1948). However, Standaert (1970, p. 79), working with *Cardisoma guanhumi,* found that "the gill lamellae stack together and fill with capillary water and appear inefficient for gas exchange." Thus, there is no guarantee of an efficient air flow between the gill lamellae. Furthermore, Cameron (1975) found no evidence for an efficient air flow in the gill chamber of *C. guanhumi* and *Gecarcinus lateralis.* These findings suggest the need for an accessory surface for oxygen uptake, such as that determined by the methylene blue test in both species.

Supporting evidence for the respiratory function detected in the branchial chamber's lining is provided from the study of the structure of the chamber's wall. The spongy aspect of the intermediate layer of the lining and the profusion of blood vessels and sinuses already suggest a respiratory function. Such characteristics of the lining's tissue are common to all species examined. However, in the absence of additional studies, it is difficult to assign a role to some structures found within the chamber's wall. The function of the globules of mucopolysaccharide observed in *Ocypode quadrata* and the freshwater crabs is to be investigated. It can be speculated that the muscular fibers found in all the species studied could be used in contracting the wall and thus facilitating the circulation of blood in the sinuses.

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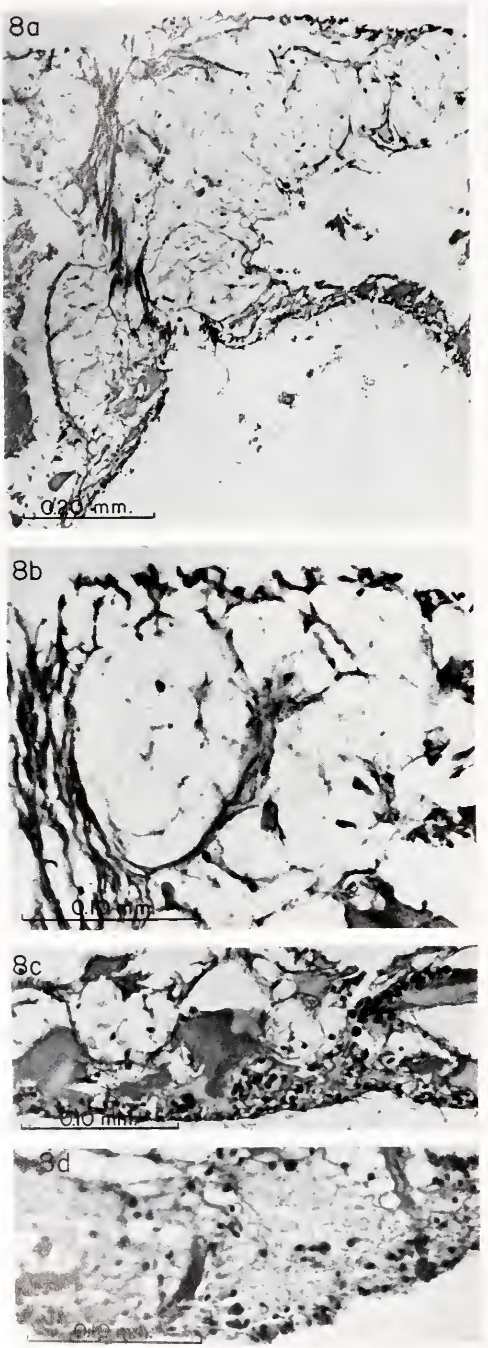
spheres and a large blood vessel. The lumen of the branchial chamber corresponds to the upper-right corner of the figure.

FIGURE 7. *Cardisoma guanhumi* Latreille: a) general view of a transverse section of the branchial chamber lining; b), c) and d) transverse sections of glandular structures from the upper layer of the lining of the branchial chamber; e) longitudinal section of a similar structure from the same region; and f) view at higher magnification of the tissue surrounding the lumen of the chamber.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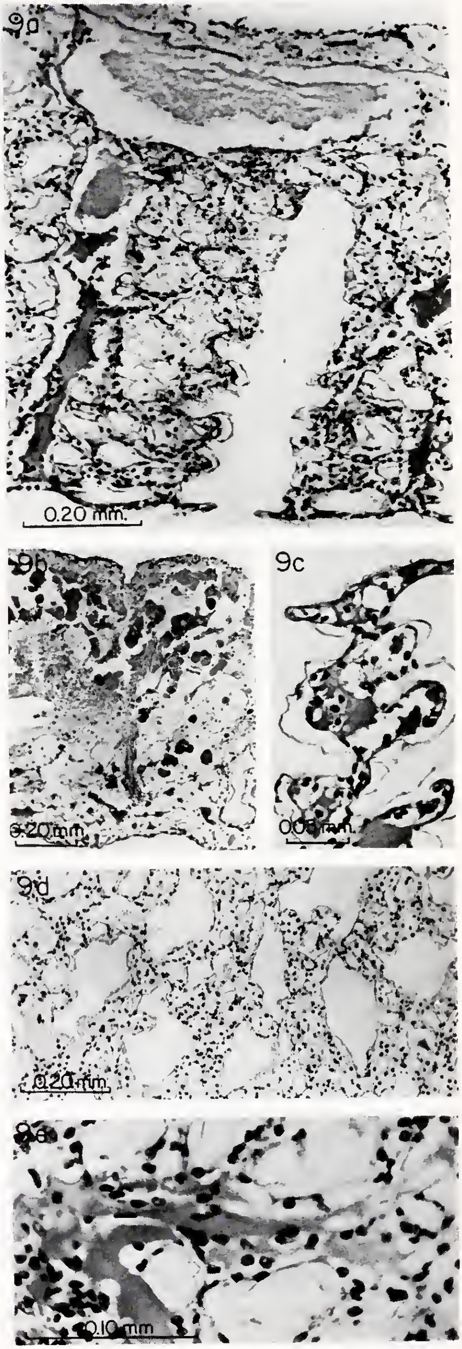
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FIGURE 8. *Gecarcinus lateralis* (Fréminville): a) low power view of a transverse section of the branchial chamber lining; b) view of higher magnification of the upper layer of the

No indication exists of the possible function of the gland-like structures found in *Cardisoma guanhumi,* unless these structures were related to the molting process, *i.e.,* the tegumental glands referred to by Dennell (1960).

Respiratory surface additional to the gill lamellae is provided by the folds in *O. quadrata.* A more efficient way for increasing surface without sacrificing space is the perforations or respiratory channels presented in the "lung" of pseudothelphusids, especially when the anastomosis of those channels, shown by sections of *Eudaniella iturbei* and *Microthelphusa simoni,* is considered.

The data collected from *Eudaniella iturbei* give some indications about the development of the perforated structure during the body growth in this species (Fig. 5, a to d). The area increases exponentially with size, while the density of perforations decreases in a similar fashion. Since the diameter of the holes increases only linearly with size of the animal, it would be reasonable to expect a similar number of perforations for the whole size range of the species. It is, therefore, not surprising that no significant relationship was obtained between the size of the animal and the estimated total number of perforations in the structure.

As the animal grows and its metabolic requirements increase, a larger surface for O_2 uptake would be needed. However, additional surface area would not have to be created by new external perforations in the structure. In this case, it appears to be produced by anastomosis of the channels in the original structure. The increased anastomosis would correspond to the linear increments in thickness of the "lung" (Fig. 5b). Data collected from other species (Table I) seem to indicate a differential increase in respiratory surface of the "lung" in relation to the species considered. The "lung" has several degrees of complexity, which appear to agree with the proposed evolutionary trend of Pseudothelphusidae (Rodríguez and Smalley, 1969). The complexity of the "lung" increased from one species to the other: it was absent in *Potamocarsinus mociñoi,* was simple and accompanied by perforations in the lining in *Pseudothelphusa jouyi,* was thicker in *P. dilatata,* and was complex in all other species which had no additional perforations in the chamber's lining (see Table I). In this latter category, the mean diameter of perforations, mean number of perforations per mm^2 and mean thickness of the "lung" were variable (Table I). However, due to lack of information for the whole size range of each species, a confident comparison among species could not be reached nor could the evolution of the "lung" in Pseudothelphusidae be traced.

The examination of the casts obtained with paraffin from the branchial chamber indicated several types of modifications of the gill chamber's shape, from the globose form found in Gecarcinidae to the pyramidal form of the Tricodactylidae. A simple lining was found in both families. A pyramidal gill chamber was also found in *Ocypode,* but with numerous folds in its lining. Pseudothelphusidae presented an elongated chamber with a globose portion in the anterior half, accom-

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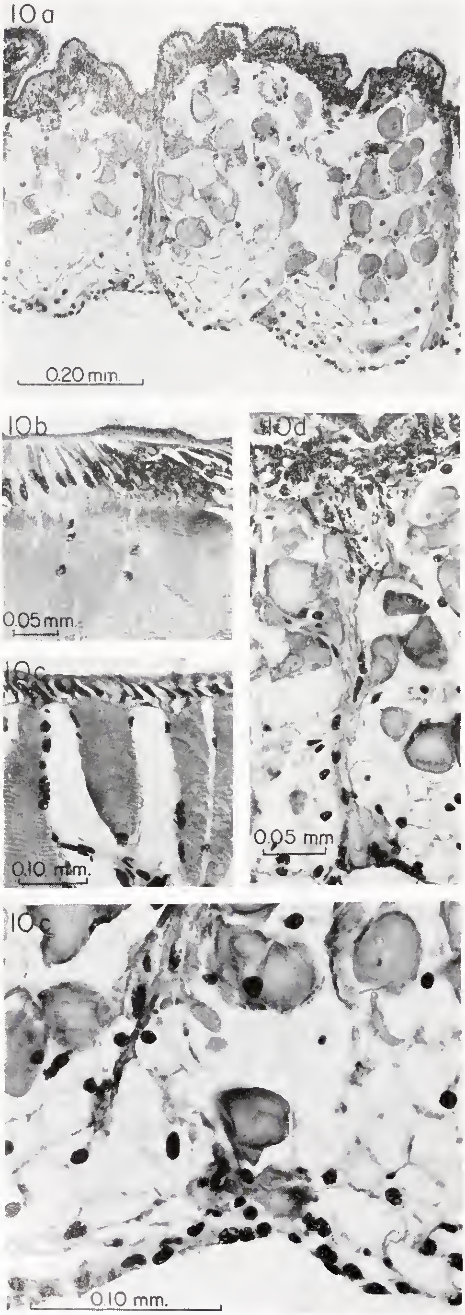
branchial chamber lining; c) and d) high power view of the lower layer of lining surrounding the chamber's lumen.

FIGURE 9. *Eudaniella iturbei* (Rathbun): a) low power view of a transverse section of the branchial chamber wall showing the lumen of one perforation; b) low power view of a transverse section of the chamber's wall from an area different from that where the "lung" is normally found; c) high power view of the tissue which forms the perforations; d) and e) view at high magnification of longitudinal sections of the "lung" of the chamber's lining.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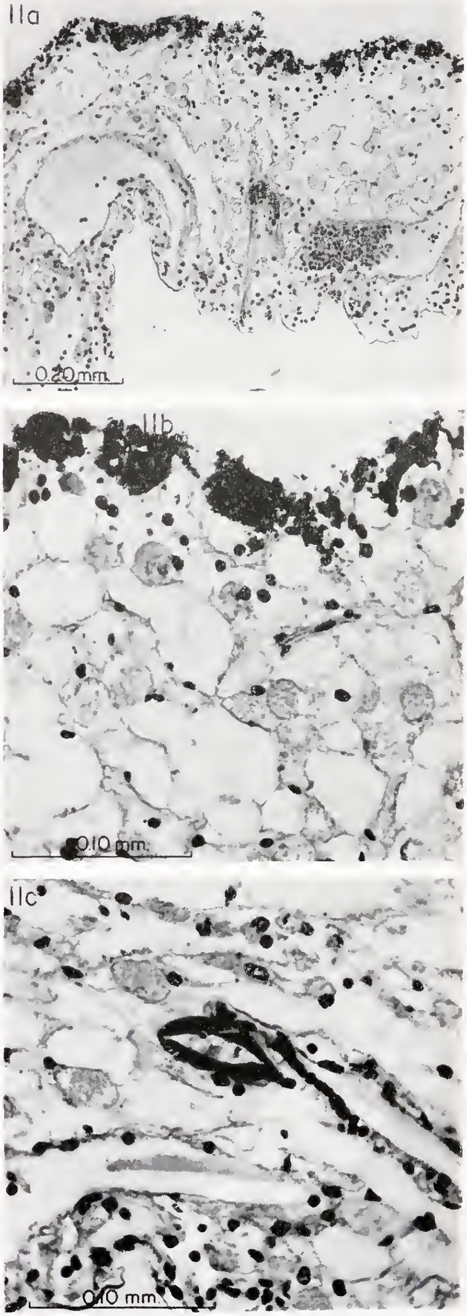
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FIGURE 10. *Valdivia venezuelensis* Rathbun: a) low power view of a transverse section of the branchial chamber wall; b) and c) from the chamber's sides, high power views of the upper layer of the branchial lining, showing insertions of muscular and connective tissue; d)

panied by the development of the "lung" described in the present work. The changes in shape of the gill chamber do not seem to be connected with the habitat of the species, but rather with the development of larger respiratory surfaces, accessory to the branchial system.

The enlargement of the branchial chamber and modifications in the structure of the epithelium covering the inner surface of the chamber, such as more abundant vascularization and increment of surface area by fold and tufts, have been interpreted as a general trend in land crabs for adaptation to life in air (Wolvekamp and Waterman, 1960). We have found evidence supporting such a generalization. At the same time, this evidence suggests that the enlargement of the gill chamber achieves the highest value in groups not showing any folding of the chamber's lining (*i.e., Cardisoma, Gecarcinus, Dilocarcinus*). As such modifications are developed (*i.e., Ocypode*), the branchial chamber volume decreases when the comparisons are made for equal body weight ranges (see Fig. 3). In general, for the species studied there is a gradual loss in volume of the branchial chamber accompanied by increasingly complex chamber walls. Thus, for a given body weight, the largest branchial chambers are those with a simple lining and the smallest, those with a complex lining. When modifications of the gill chamber walls are extreme, as in the "lung" of the Pseudothelphusidae, the ratio of branchial chamber volume to body weight becomes even more reduced. However, for all cases studied, there was an allometric relationship ($\ln y = \ln b + \alpha \ln x$) between the volume of the chamber and the weight of the animal. The volume of the gill chamber related to body weight determines three categories of chamber growth. For these three categories of chamber development, the slopes (α) of the regression lines was found to be similar ($P < 0.01$), indicating that the rate of increment of the gill chamber volume for all three groups was the same, but had a different coefficient, "b." The allometric regressions were empirically determined; they applied only to size ranges represented by the actual data (Gould, 1966). Thus, any interpretation of the coefficient "b" must be made cautiously. However, the initial values shown in the regression curves (Fig. 3) suggest different initial growth rates for each of the three categories.

During the process of land colonization by crabs, new environmental conditions generated new functional problems. In these crabs the problem of respiration was solved in three different ways, corresponding to the three categories of branchial chamber development referred to above. These categories were not apparently related to the habitat where the species normally occur. A simple lining accompanied by a relatively large chamber was found in the Gecarcinidae and Trichodactilidae. The species studied from these two families normally occur in different types of habitats (see Table I). A second category is represented by *O. quadrata*. This species has a lining with folds in a mid-sized branchial chamber. *O. quadrata* is commonly found in the same type of habitat as *G. lateralis*. Thirdly,

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high power view of the middle-area of the lining from a transverse section; and e) high power view of the lower layer of the lining which surrounds the chamber's lumen.

FIGURE 11. *Dilocarcinus dentatus* (Randall) : a) low power view of the transverse section of the branchial chamber wall; b) high power view of the upper layer of the lining; and c) high power view of the lower layer which surrounds the chamber's lumen.

the most conspicuous lining was presented by the pseudothelphusids which normally inhabit small mountain streams.

It appears that a variety of factors, in addition to change from an aquatic to terrestrial life, have affected the development of respiratory structures. For instance, *Ocypode*, the ghost crab, is well known by its quick runs on open sandy beaches; it has a small and thin carapace. Its nocturnal habitat and fast escape reaction are usually invoked as a mechanisms of defense against predation. With this adaptive strategy, an acceleration of its metabolic rate is to be expected and seems to be confirmed by physiological measurements (Standaert, 1970; Veerannan, 1974; Vernberg, 1956; Vernberg and Vernberg, 1972). The development of a greater surface available for aerial respiration in *Ocypode* may be a response to this need for higher oxygen uptake. *Gecarcinus* is found in the same general habitat, but is larger, has a more durable carapace, and is somewhat less speedy in response. Its adaptive strategy is very different from that of the more elaborate respiratory structures, having evolved towards a large gill chamber with a heavily vascularized lining devoid of folds or protuberances.

It is obvious that behavior has both a physiological and a structural basis; as the function and structure are interrelated, the integrated response of a wide variety of mechanisms is responsible for the survival of any organism. Such an integrated response becomes more evident when a comparison is established in a group of organisms from two or more types of environments, *i.e.,* terrestrial and aquatic crabs.

Pseudothelphusids are very quick in their escape reaction and aggressive behavior; they also have developed relatively strong chelae. However, their body is not necessarily robust. All the species of Pseudothelphusidae studied had the smallest branchial chamber volume in relation to body weight, thus reflecting a relatively small body form. Also, specimens of this family have been found very far from the water or under rocks of creek basins when no water was flowing (Holthuis, 1959). Furthermore, the specimens used in the present work were kept in the laboratory up to two months in containers which had just a thin layer of water. These facts indicate the ability of these organisms to survive in air for prolonged periods of time. Pseudothelphusids represent the group that has the most complex respiratory structure in addition to the branchiae, and, seemingly, the group that has achieved a more efficient use of the space of the gill chamber for respiratory purposes while at the same time remaining relatively small.

In brachyurans, the branchial chamber space becomes filled with gonads before the egg-bearing period. The availability of this space would affect the biomass directly involved in the reproductive process, *i.e.*, clutch size. Thus, the volume of the branchial chamber may be reduced by the presence of large gonads, and accessory structures may be necessary to enhance its respiratory function.

It is concluded that the development of accessory respiratory structures not only is a response to the differential availability of aquatic and aerial oxygen or to the need for oxygen uptake, but also involves a wide variety of interacting factors.

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SUMMARY

1. The structure, form and relative growth of the branchial chamber was studied in several species of amphibious crabs that inhabit marine, estuarine and fresh water. This study revealed a general trend in the solution of the problems of aerial respiration.

2. Growth of the branchial chamber volume is allometric with respect to the body weight of the crab. Data suggest the existence of three categories of complexity in the form, structure, and growth of the branchial chamber of amphibian crabs. A lessening in the chamber's volume is compensated for by an increment in the lining's surface and by development of new structures inside the chamber itself.

3. The shape of the gill chamber and the kind of respiratory structures, in addition to the branchiae, are not directly related to habitat.

4. Histological examination of the gill chamber lining showed that the structure is very similar in its general conformation in all species studied.

5. Species of Pseudothelphusidae from Venezuela mountain creeks present a perforated structure which, by qualitative tests, proved to be a respiratory organ, accessory to the gills. The location of this "lung" is on the anterolateral angle of the ceiling of the branchial chamber. The "lung" is also present in species of Pseudothelphusidae from Central and South America, including species from the Antilles. From a total of fourteen species examined from this family, only a Mexican cave dwelling species, *Potamocarcinus mociñoi,* did not have this structure.

6. The external area, density of perforations, diameter of perforations, and thickness of the respiratory structure found in Pseudothelphusidae were measured; these parameters appear to change with respect to the size of the animal and the species considered. However, the lack of data on individual species does not allow a more definite conclusion about the inter-specific variation.

7. The development of additional respiratory structures are interpreted as being a consequence of a set of environmental conditions new to the organisms as they colonize land, not just a response to the need for aerial respiration.

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OSMOREGULATION IN THE LARVAE AND ADULTS OF THE GRAPSID CRAB *SESARMA RETICULATUM* SAY[1]

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To date, most studies concerned with decapod crustacean larval responses to salinity have concentrated on the determination of optimal salinity-tolerance ranges. While these studies have often demonstrated differential salinity-tolerance ranges for larvae and adults, it is clear that those estuarine species which recruit young by retention within the estuary must possess larval stages that are physiologically adapted for estuarine existence. Although numerous studies have been concerned with osmoregulation in adult crustaceans, especially decapods (for reviews, see Krogh, 1939; Beadle, 1957; Robertson, 1960a; Lockwood, 1962, 1967; Potts and Parry, 1964; Schoffeniels and Gilles, 1970; Prosser, 1973; Vernberg and Silverthorn, 1977) and the need for studies pertaining to all stages of the life cycle has been repeatedly stressed (Prosser, 1957; Vernberg, 1967; Costlow, 1968; Vernberg and Vernberg, 1972), few data exist regarding osmoregulation in decapod larvae. Osmoregulatory adaptations employed by larvae would not, necessarily, reflect those utilized by adults. As planktonic organisms, decapod larvae are at the mercy of currents in their horizontal displacement and may be exposed, therefore, to a completely different set of environmental conditions than benthic, intertidal adult crabs. Surface-area to volume ratios and metabolic rates, both of which may be dependent on size, can interact to dictate the osmotic gradients maintained by larvae. Larvae possess functional antennal glands (Anderson, 1973) but lack gills, important sites of active ion-transport in adult decapods.

Kalber and Costlow (1966) measured the body-fluid concentrations in larvae of *Rhithropanopeus harrisii* for four zoeal and one megalopa stage and examined the effect of eyestalk removal on the osmoregulatory response. Control larvae generally remained hyper-osmotic in salinities from 10 to 30‰ and isosmotic or hyper-osmotic from 30 to 40‰ throughout all of development, except for 24 hours during the second zoeal stage, when they were isosmotic at all salinities. Kalber and Costlow (1968) and Kalber (1970) presented data on osmoregulation in larvae of *Cardisoma guanhumi, Callinectes sapidus, Libinia emarginata* and *Hepatus epheliticus.* No data were presented concerning the actual freezing point depressions of the test media and body fluids, nor were values for means and variation included. However, their graphs of the relationship between internal and external osmotic concentrations indicated the ability of *C. guanhumi* larvae to hyper- and hypo-regulate. The other species were generally hyper-osmotic in salinities of 10 to 30‰ throughout development. Larvae of *Callinectes sapidus* appeared to hyper-regulate against 40‰ as well, while larvae of *L. emarginata* and *H. epheliticus* seemed to hypo-regulate at this salinity.

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Since molting has been shown to affect the osmotic pressure of body fluids in crustaceans (Baumberger and Olmsted, 1928; Baumberger and Dill, 1928; Huf, 1933 as cited by Krogh, 1939; Prosser, Green, and Chow, 1955; Robertson, 1960b; Crowley, 1963; Lockwood and Andrews, 1969; Lindqvist, 1970; Mantel, 1975), the rapid molting cycles in decapod larvae might be reflected in cyclic changes in blood osmotic pressure during each zoeal stage throughout development. In support of this proposition, Kalber and Costlow (1966, 1968) claimed that their data indicated that the blood osmotic pressures in *R. harrisii* and *C. guanhumi* increased as each larval molt was approached and decreased within 12 hours afterward. This tendency was proposed as a mechanism to provide an osmotic gradient necessary to insure water influx at ecdysis, thereby allowing the animal to grow. Proximity to the molt was reflected in increased levels of free amino acids in larval *Callinectes sapidus* (Tucker and Costlow, 1975), but it was unclear whether the observed changes were secondary to possible hemolymph inorganic-ion changes associated with the molt or due to a more direct hormonal effect on amino acid metabolism.

In the interest of following changes in the osmoregulatory capabilities through larval development into adulthood, especially in view of the relative scarcity of data concerning osmoregulation in decapod larvae, the present study was undertaken to trace the ontogeny of osmoregulation in the estuarine, grapsid crab, *Sesarma reticulatum* Say. In addition, special emphasis has been placed on elucidating the possible effects of the molting cycle on larval osmoregulatory capabilities.

The grapsid crab *Sesarma reticulatum* Say is a common species in many salt marshes and estuaries from Massachusetts to Texas (Abele, 1973). It can be found under logs (Herreid, 1969) and burrowing into muddy areas, such as wave-cut marsh faces and higher banks of the marsh (Allen and Curran, 1974), especially where the sand content of the substratum is <10% (Teal, 1958). Throughout its range, the crab is found intertidally (Gray, 1957; Crichton, 1960; Bliss, 1968), often alongside *Uca pugnax* (Teal, 1958; Allen and Curran, 1974) and *Uca minax* (Gray, 1957). Little data on the temperature and salinity ranges experienced by *S. reticulatum* in the field are available. Teal (1959) found the crab active on cloudy days and when the tide was high. Allen and Curran (1974) found specimens in full-strength sea water behind Shackleford Banks, North Carolina, and Pinschmidt (1963), working nearby in the Newport River Estuary, found gravid females in areas where mean monthly salinities ranged from 0.3 to 33.3‰.

Development in *S. reticulatum* was examined by Costlow and Bookhout (1962), who followed the complete larval development and described three zoeal stages and one megalops stage. In another study, Costlow (1966) found that for control animals reared at 25° C in 25‰ sea water, zoeal development was completed in 9 to 11 days. The first stage lasted approximately 3 days and first crab was reached from 18 to 24 days after hatching. Larval temperature and salinity tolerances must be inferred from field studies, as laboratory data in this respect are lacking. Larvae of *Sesarma reticulatum* have been found in the plankton in the Newport River Estuary in North Carolina from May to September at temperatures and salinities ranging from 19 to 34° C and 18 to 36‰, respectively (Pinschmidt, 1963). Most larvae, however, were found between 25 and 31° C and from 25 to 36‰. Nearly all of the larvae were found in bottom samples and a differential pattern of a higher and narrower salinity range for each larval stage was noted. Tagatz (1968) found *Sesarma* larvae from 3 to 40 km above the mouth of the St. Johns River in Florida. The specimens of *Sesarma reticulatum* and *S. cinereum* were not distinguished and the larvae were grouped as *Sesarma* spp. From 3 to 11 km above the mouth of the river, where salinities ranged from 12 to 36‰, *Sesarma* spp. was second to *Uca* spp. in being most numerous. Forty km above the mouth, the salinity ranged from 9 to 27‰, and *Sesarma* spp. ranked third in abundance behind *Uca* spp. and *Rhithropanopeus harrisii*. Approximately 48 km above the mouth of the river, where salinities ranged from 0 to 11‰, the *Sesarma* spp. zoeae that were collected were dead. Dudley and Judy (1971) found *Sesarma* spp. at inshore stations outside Beaufort Inlet in North Carolina, but could find none at offshore stations. Larvae were found to be consistently more numerous at 8 m than at the surface. Sandifer (1973, 1975), working in the York River Estuary where he recorded salinities ranging from fresh to full-strength sea water, collected *S. reticulatum* larvae from June through September in salinities ranging from 2 to 20‰. Few larvae, however, were collected in waters of salinity <10‰, and most were found in the range 15 to 20‰. The total temperature range of collections was 22.8 to 27.9° C. Stage I larvae comprised approximately 73% of those collected, with stages II and III occurring in equal abundance. As Pinschmidt (1963) and Dudley and Judy (1971) found, most larvae occurred near the bottom. Nearly 75% of stage I larvae were collected in bottom samples, and virtually all stages II and III larvae were taken there. Dean (1975), working in abandoned South Carolina rice fields, found *S. reticulatum* larvae always close to the water-sediment interface.

Materials and Methods

All specimens of *Sesarma reticulatum* used in experiments were from a single population living in the banks of a muddy ditch in a part of the salt marsh bordering the Ashley River, approximately 9.6 km from the mouth of Charleston Harbor, South Carolina. The population was submerged at high tide for approximately two hours each tidal cycle. The salinity of the water, determined at various times throughout the summer of 1975, ranged from 10 to 16‰. Although *S. reticulatum* is an intertidal crab, the experimental animals were kept completely submerged to eliminate any effects of desiccation. Larvae and adults were maintained, and all experiments conducted, at 25° C under a 14L:10D light regime. Costlow and Bookhout (1962) determined that development in this crab is unaffected by photoperiod. Experimental salinities were obtained by filtering sea water from North Inlet Estuary and diluting it with distilled water or concentrating it by freezing.

Larval stages

Ovigerous females were brought into the laboratory and maintained individually in glass fingerbowls containing 25‰ filtered sea water at 25° C. Following hatching, larvae were transferred to plastic compartmentalized boxes containing

25° C, 25‰ filtered sea water for the duration of the experiments. Freshly hatched *Artemia* nauplii were added daily as a food source. The water was changed every other day as preliminary observations demonstrated no differences in survival than if the water were changed daily.

Adults

Animals were collected, returned to the laboratory and individually placed in glass fingerbowls containing 25‰ filtered sea water at 25° C. The water was changed daily, and animals were not fed during the experiments. Only males and nonovigerous females in the intermolt condition were used, as disturbances in blood osmotic pressure may be caused by the molt cycle (see introduction for references) and ovigerous animals may have altered blood osmotic pressures (Lindqvist, 1970). Although Gilbert (1959) and Tan and van Engel (1966) demonstrated different osmotic concentrations for males and females in *Carcinus maenus* and *Callinectes sapidus,* respectively, the sexes were not differentiated for data analysis in this study unless evidence appeared during the course of the experiments indicating that it was necessary to separate the results. Specimens ranged in size from 1.9 to 5.1 g, although the majority were approximately 3 g. Since Gilbert (1959) and Lindqvist (1970) have demonstrated that size may have a definite influence on blood osmotic pressure, an attempt was made to use a similar range of sizes in all experiments. Adults used in all experiments conformed to these criteria and were maintained as described.

Acclimation-time tests

When measuring the osmotic concentration of blood, it is necessary to know whether adaptation to a given salinity has been complete (Spaargaren, 1971). Therefore, the rates of salinity adaptation in the larvae and adults were determined before starting the measurements of blood osmotic concentrations.

Larvae. Stages I and III larvae, reared in 25° C, 25‰ filtered sea water, were used. Salinity-tolerance experiments have indicated that complete zoeal development is possible in a range of salinities from 10 to 40‰ (Foskett, 1977). Since both test salinities are equally different from the acclimation salinity and preliminary observations indicated that 10‰ was the most stressful salinity, determination of the time period required for the body fluids to reach osmoregulatory equilibrium following acute exposure was conducted in 10‰ test-medium. It was also assumed that stage II larvae would require an equilibrium period comparable to stages I and III.

Several larvae were placed in 10‰ test-medium and body-fluid samples taken at half-hour intervals until an equilibrium was established. At least three larvae were used at each time. Hemolymph samples were obtained as follows: the zoea was removed from the rearing medium and placed in a small pool of mineral oil, through which it was gently pushed with a pair of micro-forceps to remove some of the water clinging to the body. The zoea was removed from the mineral oil, blotted on filter paper, transferred to a small plastic petri dish and covered with mineral oil. Only larvae which displayed a beating heart and an undamaged cuticle, and to which no water still adhered, were used. Working under a dissecting scope, mineral oil was sucked up into a finely drawn-out glass capillary. The capillary was then inserted through the membrane between the carapace and first abdominal segment, slid along the underside of the carapace until the heart was reached, and the heart pierced. The hemolymph usually flowed readily into the capillary. This method is quite effective because it always provides a sufficient amount of hemolymph and one can be sure of what is actually drawn by the micropipette. Approximately 50–100 nannoliter volume of hemolymph can easily be obtained. More mineral oil was then sucked up to bracket the hemolymph sample. Freezing-point depressions were determined with a cryoscope modified after that described by Ramsay and Brown (1955) and according to the procedures they outline. Preliminary experiments revealed that, in some instances, leeching of osmotically-active substances by or from the glass capillary took place if the sample was allowed to sit at room temperature for 7 hr. In no case was leeching detected if the freezing point of the sample was determined within 2 hr of sampling. Therefore, all determinations were made within 2 hr of sampling. Usually the freezing point of each sample was measured only once.

Adults. Acclimation-rate determinations for adults were initiated by placing 20 specimens in each of the highest and lowest tolerated salinities, determined to be 50 and 5‰, respectively (Foskett, 1977). Following the procedure described by Barnes (1967), a tolerated salinity is determined as one in which at least half of the specimens had survived to the end of an 8-day period. Blood samples were taken from four animals at the beginning of the experiment and from four different animals in each of the two test salinities after 12 hr exposure. Blood was obtained by inserting the needle of a 1 cc disposable syringe into the arthrodial membrane at the base of a walking leg and withdrawing a sample. The blood was discharged onto a plastic dish and stirred slightly with the needle. In those few instances when a clot would form, only the serum was used. There is good evidence that there is an insignificant osmotic difference between whole blood and serum of crabs (Prosser *et al.*, 1955; Gross, 1964). Fifty μl of blood (or serum) were diluted with 200 ml of distilled water and the osmolality determined on a Fiske osmometer (model OS). The instrument was calibrated against standard NaCl solutions. The average of four determinations for each sample was used in computations to account for variability within the machine itself. The crabs were weighed, discarded, and the procedure repeated with fresh crabs 12 hr later and every 24 hr for a total of four days. In this manner, the amount of time required to establish osmoregulatory equilibrium was determined. It was assumed that the times required for acclimation to the intermediate salinities would be less than the longest time to either of the extremes.

Osmotic regulation in various salinities

Zoeae. Preliminary observations determined the time-course of larval development under the previously described rearing conditions. This information was necessary in order to be able to accurately predict the proper times to sample the body fluids. To trace ontogenetic changes in blood concentrations as well as changes in the blood concentrations associated with each larval molt, hemolymph was extracted and the freezing-point depressions (Δi° C) determined at ten different times during the three zoeal and one megalops stages. The sampling times were as follows: immediately after hatching, mid-way through each zoeal stage, and immediately prior to and following each ecdysis. Larvae were considered to be "immediately prior" to ecdysis if at least 50% of the larvae of a hatch had already molted into the next stage. Larvae that were sampled "immediately following" ecdysis were seen to have molted within 15 min prior to exposure to the test salinities. At each of the ten sampling times, from three to six larvae were placed in each of five test salinities of approximately 10, 20, 25, 30, and 40‰, for a period equal to or greater than the equilibrium period determined previously.

Extraction of body fluids and determination of blood freezing-point depressions (Δi° C) were carried out as already described. The freezing-point depressions of the test salinities (Δe° C) were also measured throughout the course of the experiment. The entire experiment was conducted twice on separate hatches.

Blood freezing-point depressions (Δi° C) were plotted against test-media freezing-point depressions (Δe° C) for each sampling time for each hatch. The least-squares method was used to construct regression lines through the data points, and the lines were tested for degree of linear fit by one-way analysis of variance ($\alpha = 0.05$). To determine if any significant differences existed in the osmotic responses between larvae from the two separate hatches, the regression lines representing the same sampling times for both hatches were compared for equality by one-way analysis of variance ($\alpha = 0.05$). If no difference was observed, the data from both hatches were combined for each sampling time for further statistical analysis. To compare the osmoregulatory response over the entire range of test salinities between pairs of sampling times, the regression lines of the sampling times were tested for equality of the entire lines and for equality of the slopes of the lines by one-way analysis of variance ($\alpha = 0.05$). Since differences observed between two regression lines may be caused by differences only at particular salinities, the osmotic responses at individual test salinities between sampling times were also tested for differences. Because the number of larvae used at each sampling time for each hatch was small, the Mann-Whitney test of equality of means ($\alpha = 0.05$) was used (Mann and Whitney, 1947). This was done for hatch 2 larvae only, since test media salinities varied between sampling times in hatch 1, making it impossible to use this test.

Since classification of decapod larval intermolt stages has not received the same degree of consideration as the classification of adult intermolt stages, Drach's classifications of brachyuran intermolt stages, as modified by Passano (1960), are used in figures and tables to denote the body-fluid sampling times. Larvae sampled immediately following ecdysis are designated stage A; those larvae sampled mid-way through a stage as stage C; and those larvae sampled immediately prior to ecdysis as stage D. When necessary to separate the results of the two hatches, they will be denoted as H1 and H2.

Megalops. Megalops in days 18 and 24 of larval life were subjected to 10, 20, 30, and 40‰ test media. From three to five megalops were placed in each test salinity at 25° C for a period greater than four times that determined for stages I and III larvae. Blood samples were obtained as for the zoeae.

Adults. Animals were maintained in the laboratory for six days prior to the start of the experiment as already described. In this case, animals were fed chopped fish until three days before the start of the experiment. Osmoregulatory capabilities over a range of salinities were determined by subjecting five specimens to each of approximately 5, 11, 18, 25, 37, 45, and 52‰ test media at 25° C for a period equal to the longer of the two acclimation times previously determined. The blood extractions and osmolality determinations were as described, and animals were weighed following extractions. The osmolalities of the test media were also determined. Osmolalities were converted to freezing-point depressions (Δ° C), and Δi° C, along with the standard errors of the means, plotted against Δe° C.

Results

Zoeae

The time required for the blood of mid-stage I (S1C) and mid-stage III (S3C) zoeae reared in 25‰ sea water to reach osmoregulatory equilibrium following acute exposure to 10‰ was 1 hr (Fig. 1). Therefore, before body-fluid sampling, a minimum of 1 hr was allowed for larvae in all other test salinities.

Larvae of *Sesarma reticulatum* are hyper-osmotic over the salinity range 10 to 35‰ and hyper- or isosmotic at 40‰ throughout zoeal and early megalopa life (Figs. 2–5). Hyper-regulation is most pronounced at 10‰ and decreases linearly to near isosmoticity at 40‰. The gradient maintained between the blood and external medium by individuals at 10‰ varied from 0.16 to 0.71 Δ° C, correspond-

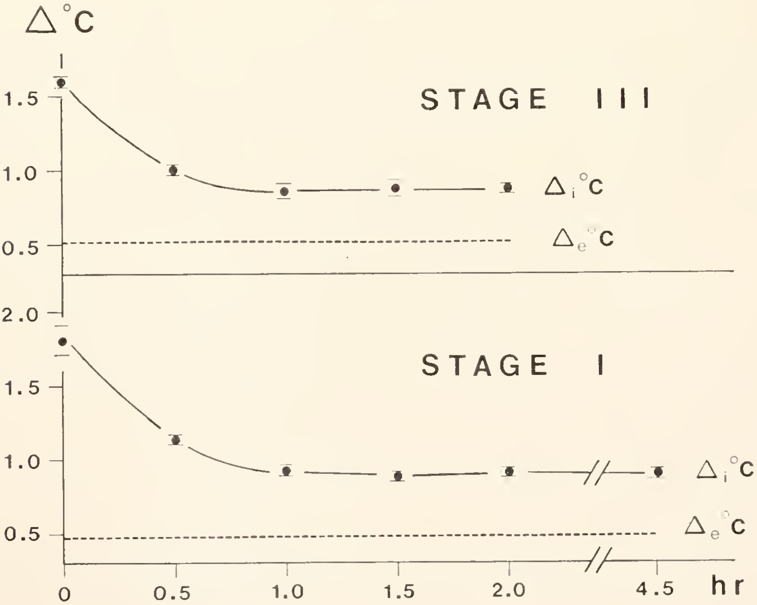

FIGURE 1. Time course of osmotic equilibrium in blood of mid-stage I (S1C) and mid-stage III (S3C) zoeae following sudden transfer from 25‰ rearing medium to 10‰; each dot represents the mean of at least three individuals; bars indicate ± s.e.m.

TABLE I

Regression lines derived from the osmotic responses of larvae sampled at eleven times during development. In those cases where no significant differences ($\alpha = 0.05$) could be detected between hatches, the regression line of the combined data, along with the P-value representing the degree of difference between the two lines, is given. See text for explanation of body-fluid sampling times.

Body-fluid sampling time	Regression lines of blood concentrations over the salinity range 10 to 40‰			
	Hatch 1	Hatch 2	Combined hatches	(P-value)
Post-hatch (S1A)	$y = 0.73x + 0.63$	$y = 0.78x + 0.60$	$y = 0.76x + 0.61$	(>0.25)
Mid-stage I (S1C)	$y = 0.74x + 0.68$	$y = 0.79x + 0.62$	$y = 0.74x + 0.68$	(>0.25)
Premolt stage I (S1D)	$y = 0.63x + 0.83$	$y = 0.81x + 0.48$		(<0.025)
Post-molt stage II (S2A)	$y = 0.87x + 0.40$	$y = 0.87x + 0.44$	$y = 0.87x + 0.43$	(>0.50)
Mid-stage II (S3C)	$y = 0.94x + 0.31$	$y = 0.72x + 0.69$		(<0.0005)
Premolt stage II (S2D)	$y = 0.87x + 0.42$	$y = 0.77x + 0.56$	$y = 0.82x + 0.50$	(>0.25)
Post-molt stage III (S3A)	$y = 0.86x + 0.35$	$y = 0.82x + 0.45$	$y = 0.84x + 0.40$	(>0.10)
Mid-stage III (S3C)	$y = 0.89x + 0.36$	$y = 0.70x + 0.67$	$y = 0.79x + 0.52$	(>0.05)
Premolt stage III (S3D)	$y = 0.78x + 0.49$	$y = 0.79x + 0.55$	$y = 0.78x + 0.52$	(>0.10)
Post-molt megalops (MA)	$y = 0.85x + 0.39$	$y = 0.88x + 0.35$	$y = 0.86x + 0.37$	(>0.75)

ing to 3 to 13‰. Regression lines depicting the relationship between Δi and Δe were tested for linearity and found to be significantly linear at all sampling times for both hatches (P-values for the ten sampling times for each hatch ranged from >0.10 to >0.95).

No significant differences (P-values ranged from >0.05 to >0.75) existed between lines representing the same body-fluid sampling time for the two hatches for all sampling times except pre-ecdysial stage I (S1D) ($P < 0.025$) and mid-stage II (S2C) larvae ($P < 0.0005$) (Table I). This indicates that the effects of variability due to inherent differences in the two hatches were insignificant except at these two times. For those times when no differences could be detected between the hatches, the data were pooled for further analysis. Therefore, the regression lines in Figures 2–5, excluding those representing the pre-ecdysial stage I and mid-stage II responses, represent the pooled data of the two hatches.

To assess the effects of molting on body-fluid concentrations, differences in the osmotic responses between consecutive sampling times were examined by testing the regression lines and the slopes of the lines for equality. Based on the hypothesis that osmotic pressures increase as ecdysis is approached, the null hypothesis was that the response at the sampling time closer to the next molt was greater than the response at the sampling time not as near the upcoming ecdysis. Therefore, the null hypothesis was that, for each larval stage, the mid-stage response was greater than the post-molt response, and the premolt response was greater than both the mid-stage response and the post-molt response of the next stage. This was done for 12 pairs of times. Differences in the lines representing the osmotic responses over the entire range of test salinities were detected for only five pairs of times.

Premolt stage I larvae of hatch 2 and mid-stage I larvae exhibited significantly different responses ($P < 0.05$). Contrary to the predicted premolt increase in blood concentration, mid-stage I larvae maintain a greater degree of hyper-osmoticity over the entire range of salinities, although the Mann-Whitney test could detect a difference only at 30‰ for hatch 2 larvae.

Premolt stage I larvae of hatch 1 and post-molt stage II larvae differ significantly ($P < 0.0005$) in their osmotic responses, although no differences ($P > 0.25$) exist in the responses of the post-molt stage II larvae and premolt stage I larvae of hatch 2. This difference is due to a greater degree of hyper-osmoticity by the premolt larvae below $\Delta e° \text{ C} = 1.65$. In more concentrated media, the post-molt larvae maintain a greater degree of hyper-osmoticity. The Mann-Whitney test indicates that a significant difference between responses exists only at 10‰ ($P = 0.05$), suggesting that the observed difference in the lines is largely due to differences in the responses at 10‰. The slopes of the lines also differ significantly ($P < 0.0005$). The slopes of the regression lines are a measure of the degree of osmotic regulation. As regulation, (*i.e.*, independence of the internal concentration to changes in the medium salinity) increases, the slope of the line will depart from 1.0 and approach 0. Therefore, the premolt larvae exhibit a higher degree of osmotic regulation than do the post-molt larvae.

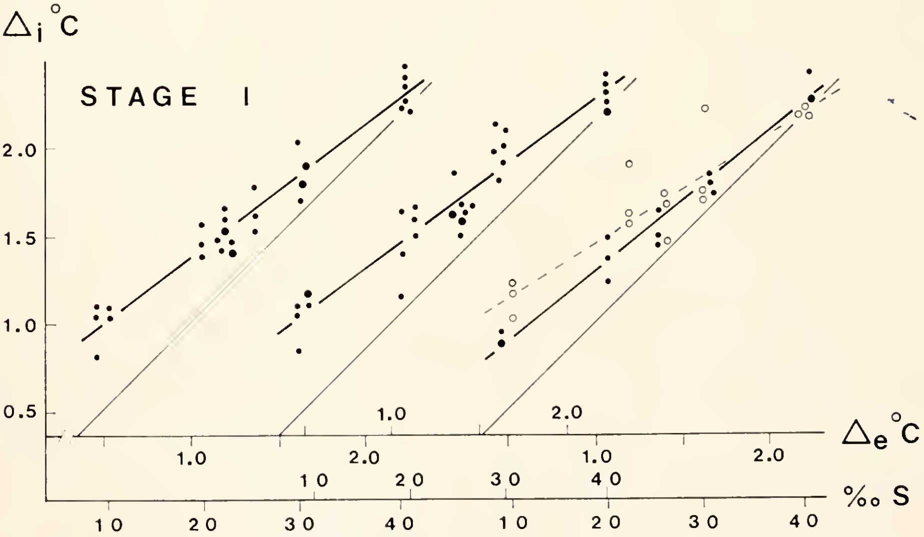

FIGURE 2. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for zoeae sampled immediately following hatching (S1A), mid-way through stage I (S1C) and immediately before first ecdysis (S1D); smaller points represent one individual, larger points represent identical responses by two individuals. Regression lines are derived from combined data of both hatches except for S1D larvae, where open circles represent responses of H1 larvae.

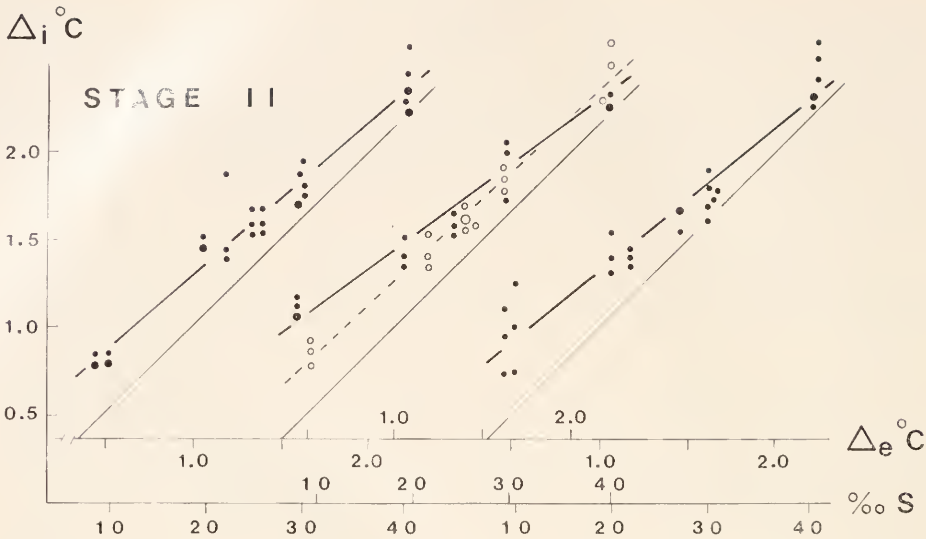

FIGURE 3. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for zoeae sampled immediately following first ecdysis (S2A), mid-way through stage II (S2C) and immediately before second ecdysis (S2D); smaller points represent one individual, larger points represent identical responses by two individuals. Regression lines are derived from combined data of both hatches except for S2C larvae, where circles represent responses of H1 larvae.

Mid-stage II larvae of hatch 2 also differ significantly ($P < 0.005$) from the post-molt stage II larval response. However, no differences could be detected between the responses of post-molt stage II larvae and mid-stage II larvae of hatch 1. Hatch 2 larvae mid-way through stage II display a greater degree of hyper-osmoticity than post-molt stage II larvae up to $\Delta e°$ C = 1.75. The Mann-Whitney test indicates a significant difference only at 10‰ ($P = 0.05$) suggesting, again, that the observed difference in the lines is largely due to differences in the response at 10‰. The slope of the regression line representing the response of the mid-stage II larvae is significantly less ($P < 0.005$) than that of the post-molt larvae, indicating a greater degree of osmotic regulation by the former.

The response exhibited by premolt stage II larvae is barely significantly different ($P < 0.05$) from that displayed by mid-stage II larvae of hatch 2, although the Mann-Whitney test could detect no differences between the responses of these two groups in hatch 2 at any salinity. The mid-stage II larvae are more hyper-osmotic up to $\Delta e°$ C = 1.98 and display a smaller slope ($P < 0.05$), indicating a slightly greater degree of osmotic regulation.

Premolt stage II and post-molt megalops larvae differ in their osmotic responses ($P < 0.05$), premolt stage II larvae displaying greater hyper-osmoticity up to $\Delta e°$ C = 1.87. Mann-Whitney tests performed on hatch 2 larvae indicate that the responses exhibited by premolt stage III larvae are significantly greater ($P = 0.05$) at 10, 20, and 25‰. The slopes of the lines are barely significantly different ($P < 0.05$), indicating that the premolt stage III larvae exhibit a slightly higher degree of osmotic regulation than post-molt megalops.

Megalops

Determination of the osmoregulatory responses of megalops was conducted for megalops in days 18 and 24 of larval life. As shown in Figure 5, megalopa remain hyper-osmotic over the entire range of test salinities, from 10 to 40‰. For both groups, the degree of hyper-regulation at 20 to 30‰ is similar to the response by the three zoeal stages at these salinities. At 10 and 40‰, however, hyper-regulation is much more pronounced in megalops when compared to zoeal stages. By day 24 a relatively high degree of homeosmosis is maintained between 10 and 20‰, in contrast to younger stages.

Adults

Measurements of the changes in blood concentration after transferring *Sesarma reticulatum* adults from 25‰ to 5 and 50‰ revealed that osmotic equilibrium was achieved immediately in 5‰ and within 72 hr in 50‰ (Fig. 6). Therefore, animals were maintained in all test salinities for 3.5 days prior to body fluid sampling.

Adult specimens of *S. reticulatum* exhibit marked capabilities of osmoregulation (Fig. 7). The blood is hyper-osmotic to the medium in the salinity range 5 to 27.5‰ and hypo-osmotic above 27.5‰. The maximum gradient sustained between the blood and external medium is 20.1‰ (Δ° C = 1.08) at 5‰. Over a range from 5 to 32‰, a high degree of homeosmosis is exhibited. Over this range, while the external medium varies approximately 26.4‰ (Δ° C = 1.43), the internal concentration varies only 2.5‰ (Δ° C = 0.14), from 25.6 to 28.1‰. At an external

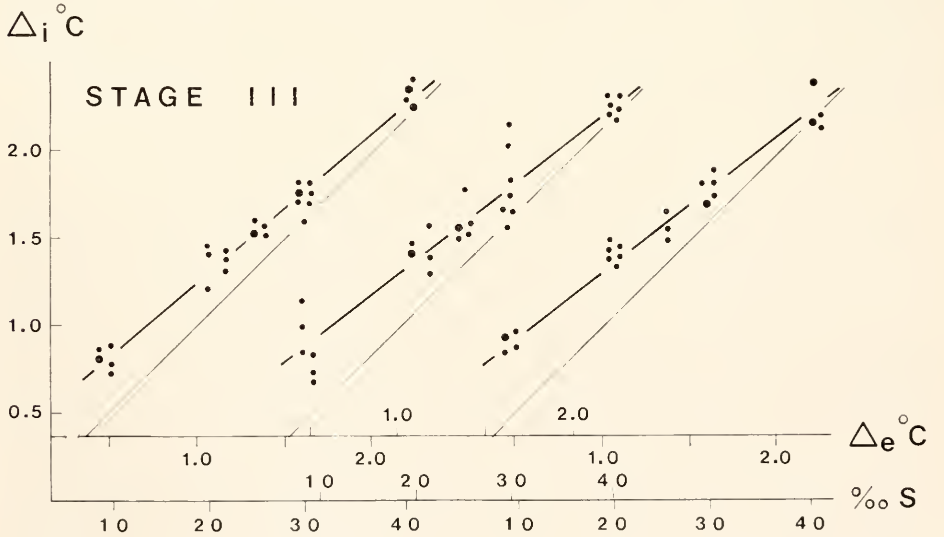

FIGURE 4. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for zoeae sampled immediately following second ecdysis (S3A), mid-way through stage III (S3C) and immediately before third ecdysis (S3D); smaller points represent one individual, larger points represent identical responses by two individuals. Regression lines are derived from combined data of both hatches.

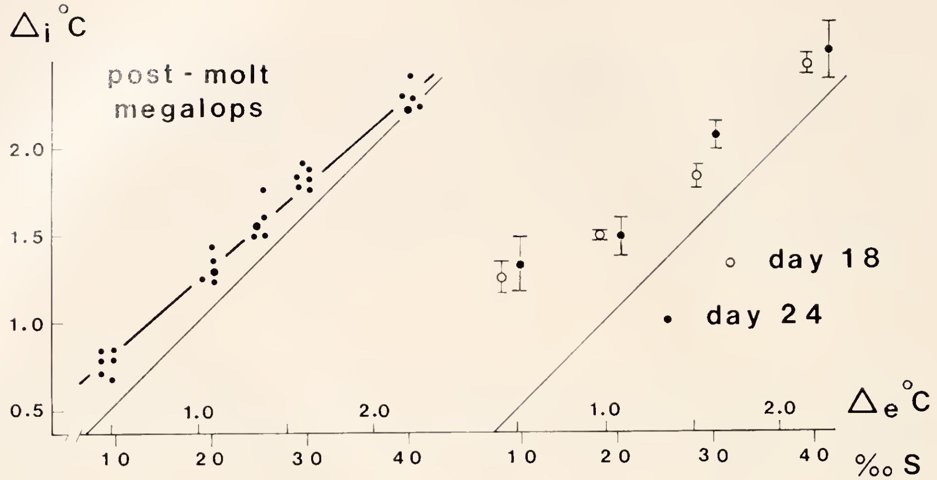

FIGURE 5. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for megalops sampled immediately following third ecdysis (MA) and at days 18 and 24 of larval life. For MA larvae, smaller points represent one individual, larger points represent identical responses by two individuals; regression line is derived from combined data of both hatches. For 18 and 24 day old larvae, each point represents the mean of at least three individuals; bars indicate ± s.e.m.

concentration of ≃32‰, this homeostasis rapidly disappears and the blood is maintained at a nearly constant difference (≃5.6‰) less than the external medium up to 45‰. Maximum hypo-osmoticity is displayed at 52‰ sea water, where the sustained gradient is ≃7.9‰. No significant differences between males and females or between animals of different sizes were detected.

DISCUSSION

The pattern of osmoregulation in larvae of *S. reticulatum* is apparently established before hatching and varies little throughout larval development. The concentration gradient sustained between blood and medium is maximum at 10‰ and decreases linearly to near isosmoticity at approximately 40‰. No significant degree of homeosmosis is exhibited over any part of the salinity range 10 to 40‰, indicating a relative conformity between salinity variations and changes in body fluid concentrations. While maintenance of a blood concentration hyper-osmotic to dilute media is a typical response of estuarine and coastal species, larvae of *Sesarma reticulatum* are unusual in maintaining blood osmotic pressures greater than the external medium in salinities as high as 40‰ during parts of larval development. Larvae of another estuarine crab, *Rhithropanopeus harrisii,* also display this specialized ability to hyper-regulate against particularly high salinities throughout most of larval life (Kalber and Costlow, 1966). The degree of hyper-regulation maintained at other salinities is also similar to that of *S. reticulatum* larvae. Larvae of *Callinectes sapidus* also hyper-regulate in 40‰ sea water, but only during the first three zoeal stages and day three of megalops. At other times, they are isosmotic or hypo-osmotic at this salinity. The gradient sustained between blood and medium at 10‰ is less than that for *S. reticulatum.* During the seventh zoeal stage, the ability to osmoregulate is apparently lost, and the larvae become isosmotic at all salinities (Kalber, 1970). Larvae of the land crab, *Cardisoma guanhumi,* are usually hypo-osmotic at 40‰. During the third zoeal stage they become hypo-osmotic in salinities as low as 20‰ as well. The gradient maintained between blood and medium at 10‰ by larvae in the first two zoeal stages is similar to the response by *S. reticulatum* larvae. In later stages, the sustained gradient is considerably diminished (Kalber and Costlow, 1968). Osmoregulation in larvae of two polystenohaline crabs, *Hepatus epheliticus* and *Libinia emarginata,* resembles that of typical estuarine species. To the first day of the third zoeal stage, larvae of *H. epheliticus* remain hyper-osmotic in salinities up to approximately 35‰ and hypo-osmotic in 40‰ sea water. As in *S. reticulatum,* the osmotic response varies little throughout development. Larvae of *Libinia emarginata,* on the other hand, begin zoeal life isosmotic to the medium in all salinities, gradually develop hyper-regulation in salinities less than 27 to 30‰ during the middle of larval life, and again become isosmotic in the third day of megalops development (Kalber, 1970).

Kalber and Costlow (1966, 1968) and Kalber (1970) allowed a 1 hr period of adjustment for larvae of *R. harrisii* and 2 hr for larvae of *C. guanhumi, C. sapidus,* and *L. emarginata* following exposure to test salinities before sampling the body fluids. However, no data showing the time-course of osmotic equilibrium were presented to confirm that equilibrium had, indeed, been attained within those time periods. The change in blood concentration after a change in the salinity of the medium is important in the ecology of a species (Gross, 1957; Kinne, 1963, 1967;

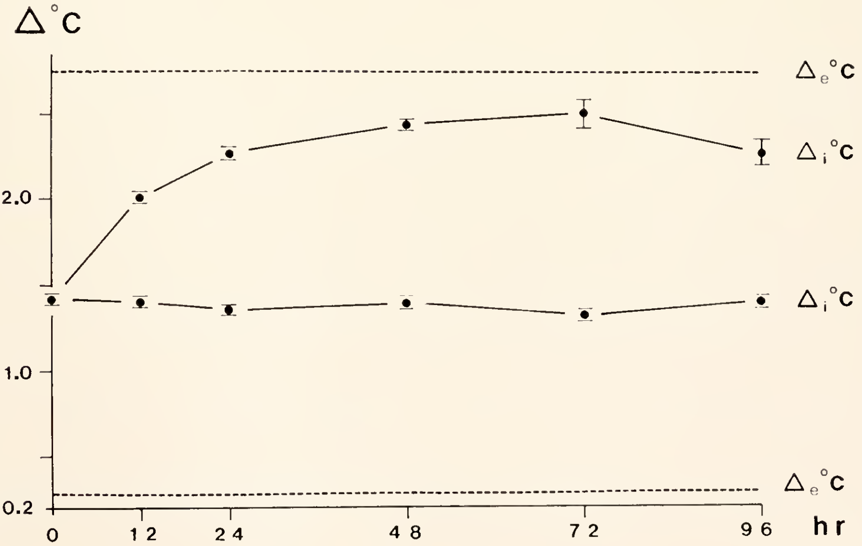

FIGURE 6. Time course of osmotic equilibrium in blood of adult *Sesarma reticulatum* following sudden transfer from 25‰ acclimation medium to 5 and 50‰; each dot represents the mean of four individuals; bars indicate ± s.e.m.

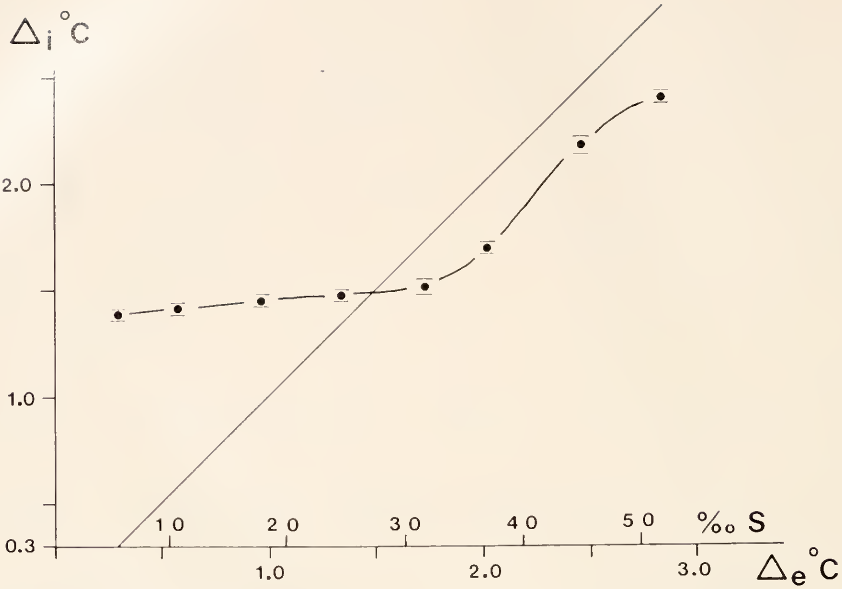

FIGURE 7. Osmotic concentration of the blood of adult *Sesarma reticulatum* as a function of osmotic concentration of the medium; each point represents the mean of five individuals; bars indicate ± s.e.m.

Spaargaren, 1971). Tidal rhythms and sudden heavy rainfalls cause rapid salinity fluctuations to which estuarine organisms are often exposed. Rapid attainment of blood osmotic equilibrium is clearly advantageous to estuarine organisms. However, osmotic adjustments which are too fast will cause blood concentrations to fluctuate back and forth in synchrony to external salinity fluctuations with possible detrimental effects (Kinne, 1967). Results obtained in the present study demonstrate that stages I and II larvae of *S. reticulatum* adjust to salinity changes within 1 hr of exposure. Since the blood concentrations in *S. reticulatum* larvae follow closely changes in the external salinity, the fact that such adjustments are attained rather rapidly suggests that the body fluids are in a constant state of osmotic flux in variable-salinity estuaries. At the tissue level, therefore, not only must the cells exhibit tolerances to wide ranges in concentration, but adjustment of intracellular concentration must be rapid to limit large fluxes of water between blood and cells.

No consistent degree of variation between individuals from the same hatch and same test salinities whose body fluids were sampled at the same time were noted for *S. reticulatum* larvae. Variation in the osmotic responses of individual larvae whose body fluids are sampled at the same time can be caused by differences in size, metabolic rates, or general healthiness. It might also be expected that some degree of variation can exist among individuals as a result of their differing genetic capabilities for osmoregulation (Barnes, 1968). Such differences could exist between individuals hatched from the same female and between individuals hatched from different females. This variation can have important evolutionary and ecological implications in variable-salinity environments (Barnes, 1968). At certain stages of larval development, more variation was exhibited in moderate than extreme salinities, while the converse was true for larvae sampled at other times. In most instances, no significant differences in the osmotic responses were exhibited by larvae from separate hatches. Only for pre-ecdysial stage I and mid-stage II larvae did significant differences exist between regression lines derived for each hatch. At both of these sampling times, the differences between the hatches were due primarily to differences in the responses at the salinity extremes 10 and 40‰. Differences between the responses of individuals from the two separate hatches at the other sampling times were also due, in large part, to differences in the responses at the extreme salinities. Under short-term, severe salinity-stress, such individual variation could insure that at least some proportion of the larval population would survive, and selection for individual larvae possessing the greatest capabilities for osmoregulation might result in further penetration into the estuary (Barnes, 1968).

In marked contrast to the osmoregulatory responses of the larvae, adult specimens of *S. reticulatum* display impressive powers of hyper- and hypo-regulation over a wide range of salinities. The response is similar to those displayed by some prawns and most crabs of the grapsoid families (see Lockwood, 1962; Panikkar, 1950; and Prosser, 1973, for lists of species). The maximum gradient maintained between blood and medium concentrations by adults in dilute sea water is much greater than for the larval stages and is comparable to that displayed by *Uca crenulata, Pachygrapsus crassipes, Ocypode ceratophthalma* (Jones, 1941; Gross, 1964), and two *Sesarma* species from South Africa (Boltt and Heeg, 1975). At 5‰ there is still no drop in the osmotic pressure to indicate breakdown of the osmoregulatory ability. The degree of hypo-osmoticity remains nearly constant in salinities above 32‰ at a concentration intermediate to that displayed by other hypo-regulators examined (see Gross, 1964; Gross, Lasiewski, Dennis, and Rudy, 1966; and Barnes, 1967).

In light of the differences in the osmoregulatory responses between larval and adult forms, and since adult specimens of *Sesarma* occupy habitats in low-salinity environments in which complete larval development is not possible (see Foskett, 1977), the osmoregulatory capabilities necessary for such penetration into estuaries must unfold during intermediate developmental stages. Although laboratory-reared megalops appear to be more tolerant than zoeal stages (Foskett, 1977), it is apparent that even by late megalops the adult osmoregulatory response is still not attained. Examination of osmoregulation in early juvenile crab stages may reveal osmoregulatory responses that are transitional between the larval and adult forms. This appears to conform to results obtained for other larvae examined. Pearse (1932) found adult specimens of *Cardisoma guanhumi* to be hypo-osmotic in 36‰ sea water, and Quinn and Lane (1966) have demonstrated that *C. guanhumi* is able to regulate Na^+ and K^+ at high levels after seven days exposure to distilled water. Apparently, *C. guanhumi* is a hyper-hypo-regulator. Although Kalber and Costlow (1968) found later stages of *C. guanhumi* larvae to be hypo-osmotic in higher salinities (30 and 40‰), the fact that they were also hypo-osmotic at 20‰ and isosmotic at 10‰ indicates that the adult response is still not attained by the end of zoeal development. Larvae of *Rhithropanopeus harrisii* in the final zoeal stage osmoregulate similarly to earlier larval stages (Kalber and Costlow, 1966), but unlike adults (Smith, 1967). *Hepatus epheliticus,* an osmoconformer as an adult, remains hyper-osmotic throughout zoeal life (Kalber, 1970). Presumably, larvae nearing megalops and settling crab stages gradually lose this ability, but no data were presented to verify this claim. Larvae of *Libinia emarginata* attain the adult response by gradually becoming isosmotic with the external medium throughout early megalopa (Kalber, 1970). However, since first stage zoeae are also generally isosmotic with external salinities, it cannot be discerned if isosmoticity in later stages simply represents the osmoregulatory pattern of larvae for that stage of development, independent of the future adult response, or if such isosmoticity truly represents transition to adult patterns of osmoregulation. Likewise, megalops of *Callinectes sapidus* osmoregulate similarly to the adults (Ballard and Abbott, 1969; Kalber, 1970) but, again, early zoeal stages also display a similar response. To summarize, then, there appears to be no clear trend toward development of adult osmoregulatory patterns toward the end of larval life. Although later stages of *Callinectes* and *Libinia* display a response similar to adults, even the early stages exhibit responses much like the adults. In forms such as *Sesarma* and *Cardisoma,* where adults display hypo- as well as hyper-regulation and which exhibit significant degrees of homeostasis, the adult osmoregulation pattern is still not established by late megalops. It is interesting, however, that *Cardisoma* larvae possess the ability to hypo-regulate while *Sesarma* larvae do not.

For most species examined thus far, there appears to be a general larval trend of hyper-osmoticity in salinities encountered in nature for most or all of larval life. The significance of this observation is not readily obvious. Kalber and Costlow (1966) and Kalber (1970) propose that hyper-osmoticity in all salinities is a mechanism to provide an osmotic gradient necessary to insure water influx at ecdysis. Kalber and Costlow (1966) sampled daily the body fluids of *R. harrisii* larvae throughout zoeal development and claimed to detect increased hyper-regulation immediately before and up to 12 hr after each molt. Since a minimum of data was chosen to be presented and differences between regression lines and responses at individual salinities not statistically analyzed, it is difficult to see clearly that such a pre-ecdysial rise in blood osmotic pressure is a general larval trend. Examination of the available data indicates that hyper-regulation in *R. harrisii* was more pronounced immediately prior to ecdysis only at 40‰, a salinity which is probably never encountered by larvae of this species. Data for other species (Kalber and Costlow, 1968; Kalber, 1970) provide no support for the hypothesis that increased hyper-osmoticity is necessary for water influx at the molt. To actually test the validity of such a hypothesis, the osmotic responses of *S. reticulatum* larvae were determined immediately before and after ecdysis and mid-way through each stage. If Kalber's hypothesis is correct for *Sesarma* larvae, the body-fluid concentrations immediately before ecdysis should be greater than those immediately after ecdysis and mid-way through the stage preceding the molt. The results obtained reveal no consistent tendencies of larvae to increase hyper-regulation over the entire range of test salinities immediatly before ecdysis compared to other times.

The normal physiology of crustaceans is continually dominated by the molt cycle. Internal changes occurring in the integumental tissues, hepatopancreas, urine and blood (Bursey and Lange, 1971), as well as metabolism, behavior, reproduction and sensory acuity are all affected by the periodic replacement of the integument (Passano, 1960). Since the molting cycle is so short for crustacean larvae, the changes associated with such growth are magnified in their rapidity. In adults, the various phases of the molting cycle are often reflected in changes in blood composition. As a result, various physiological problems may arise due to variations in ionic ratios and total ionic concentrations, and from dilution and changes in body surface permeability (Lockwood, 1967). Baumberger and Olmsted (1928), working with *Pachygrapsus,* were the first to notice differences in the blood osmotic pressures between different stages of the molt cycle. They noted an increased blood osmotic pressure immediately before ecdysis and a subsequent drop following the molt. They postulated that such a rise before ecdysis was responsible for water uptake necessary for growth. However, Robertson (1960b) has criticized the techniques utilized and the significance of their findings. In another study, Baumberger and Dill (1928) found an increase in blood osmotic pressure during the act of molting in *Callinectes* and, again, postulated that such a rise could account for water uptake at the molt. Since then, others have noted similar pre-ecdysial rises in blood osmotic pressure (Parry, 1953; Robertson, 1960b; Lockwood and Andrews, 1969) but have demonstrated that such rises are insufficient to account for the water uptake at the molt. There are exceptions to the general rule of premolt increases and subsequent post-molt decreases in blood osmotic pressures. Crowley (1963) found an average 12 per cent decrease in total cation concentration in the premolt period as compared to the normal late intermolt levels in several species of crayfish. Lindqvist (1970) found two species of terrestrial isopods which displayed lower blood osmotic pressures during molting than did nonmolting animals. Although Parry (1953) found a premolt rise in blood osmotic concentrations in *Ligia oceanica,* there was no indication of a sudden uptake of water after the molt. Post-molt animals had higher blood osmotic concentrations than premolt animals and even animals sampled four days after the molt showed no subsequent drop in osmotic pressure. Since cells are generally assumed to be isosmotic with the blood (Schoffeniels and Gilles, 1970) and organic compounds account for about half of the total intracellular osmotic pressure in crustaceans, changes in hemolymph concentrations associated with the molt should be reflected in changes in the levels of these organic constituents, especially the free amino acids. Contrary to such expectations, Dall (1975) found no significant changes in the levels of leg muscle and blood ninhydrin-positive substances during the molt cycle in the rock lobster, *Panulirus longipes.* In *Gecarcinus lateralis,* the total free amino acid content in the claw muscle decreased as the molt was approached and increased during the post-molt period (Yamaoka and Skinner, 1976). The decreased premolt concentrations could not be explained by dilution of the body fluids and tissue hydration.

Clearly, the changes in the blood resulting from the molting cycle vary between species and may be complicated by such factors as water absorption during and after the molt, the amount of stored substances in the tissues, and the relative requirements for these stores in providing energy and material for the new exoskeleton (Florkin, 1960). With respect to *S. reticulatum* larvae, an osmotic gradient between blood and medium may well be necessary to insure water influx at the molt. However, there would seem to be little need to raise the pre-ecdysial blood concentration, since the blood is already hyper-osmotic to the entire range of salinities in which the larvae can survive. The adults of many species display increased concentrations of calcium, proteins, and other ions prior to molting. Most often, the increases are attributed to resorption of these constituents from the old exoskeleton. Therefore, in large part, it is the heavily calcified nature of the exoskeleton of adults which causes large-scale changes in the blood at the molt. The various constituents are resorbed, stored in, and transported by the blood, necessitated by the fact that large amounts of these substances must be readily available for deposition in the new exoskeleton. For larval stages, which lack a heavily calcified exoskeleton, there is no need to resorb various constituents from the old exoskeleton, because sea water probably contains sufficient amounts for deposition in the new one. Larvae would not be expected, therefore, to display increased concentrations of these constituents prior to molting.

Robertson (1960b) has shown that the water taken up at the molt is isosmotic to the medium. Since larvae of *Sesarma* are hyper-osmotic, the rapid uptake of sea water may be expected to dilute the blood during and after the molt. As previously discussed, changes in larval blood concentrations follow changes in the medium concentrations so closely and so quickly (within 1 hr), that a rapid turnover of salts and water between larva and medium is indicated. The mechanisms responsible for maintenance of hyper-osmoticity over the entire range of test salinities are designed to operate successfully despite such a rapid turnover. That no consistent post-molt dilutions were detected for *Sesarma* larvae may be due to the fact that the salt-uptake mechanisms are necessarily adapted to cope with rapid turnovers of water and salts, such as occurs during the molting process. Such osmoregulatory mechanisms are probably under hormonal control. A quantity of evidence supporting the existence of neuroendocrine control of salt and water regulation in adult crabs has emerged (for a review see Kamemoto, 1976). With respect to larval stages, eyestalk removal promotes greater size increases at the molt for *Callinectes* larvae (Costlow, 1963); and Kalber and Costlow (1966) found that larvae of *Rhithropanopeus* with eyestalks removed 12 hr after the first molt lose the ability to hyper-regulate in salinities below 30‰, while increasing hyper-regulation at 40‰. A day later, however, the eyestalkless larvae become hyper-osmotic in salinities ranging from 10 to 40‰ but again lose the ability to hyper-regulate in salinities below 30‰ following the second molt. Therefore, while the trend of decapod larvae to hyper-regulate over the range of salinities encountered in nature may be related to a necessity to maintain an osmotic gradient in order to insure water influx at ecdysis, there is no evidence that increased hyper-osmotic gradients are necessary at the time of molt. Instead, it would appear that neuroendocrine controls are probably more important in insuring water uptake at the molt, possibly through regulations of the extent of body-surface permeability to salts and water, as demonstrated for *Gecarcinus lateralis* (Mantel, 1968).

Since many estuaries are characterized by a two-layered, stratified circulation in which deeper, landward-flowing salt water is overlain by seaward-flowing fresh water the significance of the general larval trend of hyper-osmoticity in salinities normally encountered in nature may be understood if hyper-osmoticity is considered as a mechanism to increase the density of the larva, enabling it to remain close to the bottom, thereby helping to insure retention within the estuary. Since larvae lack a heavy exoskeleton, hyper-osmoticity in all salinities may also be necessary to provide turgor pressure to insure integrity of the thin larval cuticle. This would explain why larvae, including older megalops, display a different osmoregulatory response from adults and suggests that the adult response will be attained only with the appearance of a rigid exoskeleton during the early juvenile crab stages.

SUMMARY

1. Blood osmotic concentrations in the three zoeal stages, megalops stage, and adults were determined over a wide range of salinities for the estuarine, grapsid crab, *Sesarma reticulatum* Say.

2. Larvae are hyper-osmotic over the salinity range 10 to 35‰ and hyper- or isosmotic at 40‰ throughout zoeal and early megalopa life. Older megalops display increased hyper-osmoticity at 10 and 40‰, compared to earlier zoeal stages.

3. Adult specimens of *S. reticulatum* are hyper-osmotic in the salinity range 5 to 27.5‰ and hypo-osmotic in salinities >27.5‰. The adult response is apparently attained during early juvenile crab stages.

4. The effects of molting on blood osmotic concentrations is discussed. The rapid molting cycles of the larvae do not affect the blood osmotic concentrations.

5. The general larval trend of hyper-osmoticity over wide ranges of external salinities may serve to increase the density of the larva, helping to promote retention within the estuary. Hyper-osmoticity may also act to provide turgor pressure to insure integrity of the thin larval cuticle.

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EVIDENCE FOR AN ADIPOKINETIC FUNCTION OF THE RPCH ACTIVITY PRESENT IN THE DESERT LOCUST NEUROENDOCRINE SYSTEM[1]

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Crustacean red pigment concentrating hormone (RPCH) is an invertebrate neurosecretory hormone that has been fully characterized and synthesized (Fernlund and Josefsson, 1972), and considerable data are currently available concerning this octapeptide (Josefesson, 1975; Carlsen, Christensen, and Josefsson, 1976; Christensen, Carlsen, and Josefsson, 1977). By contrast, although it has long been known that RPCH-like activity is present in the cephalic neuroendocrine systems of many insects (Hanstrom, 1940; Brown and Meglitsch, 1940; Thomsen, 1943), prior to the initiation of this study little was known of the function or chemistry of the insect material (see Knowles, Carlisle, and Dupont-Raabe, 1955). In view of the availability of synthetic RPCH, the relative ease of the standard *Leander* bioassay for this hormone, and an understanding of RPCH chemistry, it was felt that studies designed to examine the possible function and chemistry of the insect red pigment concentrating activity (RPCA) could be of special interest. Such studies were therefore begun in Copenhagen in the fall of 1975. Data dealing with the presence of RPCA in several insects and the probable function of RPCA in the desert locust *Schistocerca gregaria* are discussed below. These data confirm and extend reports, published after the completion of this study, dealing with the structure and biological activity of the locust adipokinetic hormone (Stone, Mordue, Batley, and Harris, 1976; Mordue and Stone, 1976).

MATERIALS AND METHODS

Animals

Specimens of *S. gregaria*, reared from eggs provided by the Centre for Overseas Pest Research, London, England, were maintained under crowded conditions in cages with a 16 hr daily photophase and a temperature of about 30° C, and fed frequently on lettuce and bran. Assays for RPCA in fifth instar organs used animals in postmolt (0–1 day after ecdysis), intermolt (4–5 days after ecdysis; no internal apolysis), and premolt (9–10 days after ecdysis; obvious advanced new cuticle formation). Adult organs were assayed 1, 10 and 20 days post-fledging; only the 20 day animals were from actively mating populations. Most of the functional studies used adults of both sexes and all ages; no sexual or age-related

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differences in response were noted. Neck-ligatured specimens of *S. gregaria* were prepared by tightly tying a thin string around the neck just posterior to the head capsule and then cutting off the head just anterior to the ligature.

Specimens of *Tachycines asynamorus* and an unknown *Dytiscus* sp. were collected near Copenhagen. Other insect species were obtained from Danish laboratory cultures (*Tenebrio molitor, Periplaneta americana,* and *Calliphora erythrocephala*), or from the Centre for Overseas Pest Research (*Locusta migratoria*), imported from California (*Danaus plexippus*). These insects were generally adults of mixed ages and sexes. However, both large nymphs and adults of *T. asynamorus,* and a mixture of late premolt fifth instars and newly fledged adults of *L. migratoria* served as donors. Organs from all of these species, except the latter (see below), were collected soon after their arrival in the laboratory. The crustaceans, *Leander adspersus, Crangon vulgaris,* and *Carcinus maenas,* were collected near the Danish coast and maintained for variable periods in artificial sea water at 10° C.

Extractions

Brains and corpora cardiaca (CC) from *S. gregaria* and other insects were normally collected by dissection at intermediate magnification (about 20×), using #5 jeweler's forceps and iridectomy scissors, but dissection of specific regions of these organs in *S. gregaria* used higher magnifications. The pars intercerebralis was roughly separated from the remainder of the brain by cutting a deep V-shaped wedge out of the mid-dorsal region of each brain; cuts were initiated just lateral to each antennal nerve. Great care was used in attempts to separate the glandular region of the CC from the storage lobe (Highnam, 1961), but total separation of these two lobes was, as in other such studies (Goldsworthy and Mordue, 1974), probably not accomplished. Dissections were conducted using chilled insect saline.

For RPCA assays on organs from the early portion of the *S. gregaria* life cycle, previously frozen and lyophilized whole heads of the first three instars and previously frozen brains and CC dissected from fourth instars were used; all organs from these young animals, of undetermined sex and molt cycle stage, were collected at the Centre for Overseas Pest Research and transported frozen to Copenhagen. Similarly, specimens of *L. migratoria* CC were collected in London and transported frozen to the Panum Institute. Dissections were not attempted on *T. molitor* or *C. erythrocephala;* instead, heads were lyophilized and stored at −20° C until extraction. All other extracts were prepared from freshly dissected material.

For the studies on RPCA content during the *S. gregaria* life cycle, extracts of first, second, and third instar heads were made by pulverizing lyophilized material in a small mortar and extracting the powder obtained at a concentration of ten heads/ml (first instar) or five heads/ml (second and third instars). Extracts of fourth instar brains and CC were made at concentrations of four brains/ml and 2 CC/ml. Extracts of fifth instar and adult whole brains and CC were made at 2/ml, and 1/ml, respectively; 4 fifth instar or adult brains or 4 CC were pooled for each extraction. Brain and CC regions were usually extracted at ten brain regions and 0.5 CC regions/ml. Extracts of other insects used similar techniques, with concentration ranging from 1 CC/ml (*L. migratoria*) to 20 heads/ml (*C. erythrocephala*). For positive controls during the functional studies on *S. gregaria*, CC extracts of various concentrations were lyophilized and the residues dissolved at a concentration of 1 CC/50 μl.

For all extracts, a measured volume of glass-distilled water was placed in a Potter- Elvejem homogenizer and chilled on ice. Either fresh organs rapidly dissected from severed heads, lyophilized powder, or frozen organs were then placed directly into the ice cold solvent. Samples were then immediately homogenized for 1 min at slow speed with a motor-driven pestle, replaced in the ice bath for 1 min, and rehomogenized for 1 min at high speed. Crude extracts were transferred to centrifuge tubes and boiled for 5 min. After boiling, the extracts were centrifuged for 15 min at 12,000×*g* in a Sorvall RC2-B superspeed centrifuge maintained at 4°C. The resulting supernatants were removed and frozen at −20° C until assayed.

Hemolymph for RPCA analysis from selected *S. gregaria* stages was squeezed from a severed rear leg, collected in microcapillary tubes, diluted 1:1 with 1.6% NaCl, boiled 1–3 min, centrifuged for 30 min at 12,000×*g* in the above centrifuge, and the supernatant frozen for subsequent assay.

Assays

RPCA was assayed on eyestalkless specimens of *L. adspersus* using a (2 + 2) point parallel line assay with five animals at each dose (Fernlund, 1968). Synthetic RPCH was used as standard in concentrations of 2.48×10^{-4} and 0.82×10^{-4} μg/ml. This method is referred to below as the *Leander* assay. One unit of RPCH is defined as 0.62×10^{-4} μg of the synthetic hormone.

The effects of RPCH on *S. gregaria* heart rate and malpighian tubule activity were assayed as described by Mordue and Goldsworthy (1969). Total hemolymph lipids were routinely measured by a colorometric method (Goldsworthy, Mordue, and Guthkelch, 1972) using a total serum lipid kit obtained from Boehringer Corp. Ten μl of *S. gregaria* hemolymph (collected into a microcapillary from a small puncture near a rear leg), and 25 μl of *L. adspersus* hemolymph (removed from the pericardial cavity with a Carlsberg micropipette) were routinely used for lipid determinations.

Hormones and analogues

The synthetic RPCH, pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2, used during this study was that mentioned above; it was available at the Panum Institute. The analogues used have recently been synthesized in the same laboratory (Christensen, Carlsen, Josefsson, 1977; Christensen, Carlsen, and Josefsson, in preparation). They include: first, the tyrosine analogue, in which phenylalanine is replaced by tyrosine; secondly, the tetrapeptide, pGlu-Pro-Gly-Trp-NH_2; thirdly, the heptopeptide amide, in which the C-terminal tryptophan amide is removed and glycine amide is the C-terminal; and fourthly, the heptapeptide, with glycine as the C-terminal amino acid.

Hemolymph lipid analysis

In one experiment, a pooled sample of 100 μl hemolymph from several experimental or several control animals was directly added to 1 ml chloroform: methanol 2: 1, v/v), and extracted for 1 min. After separation, the lower phase was collected, and the upper extracted again with 500 μl chloroform: methanol (1:2, v/v) for 1 min. After mild centrifugation, the upper phase of this extraction was combined with the previous lower phase. The combination phases were then washed with 300 μl 0.1% NaCl, vortexed 1 min, centrifuged, and the bottom phase collected. Each sample was then evaporated under nitrogen and frozen for subsequent analysis by thin layer chromatography (TLC) and for gravimetric total lipid determination.

TLC was performed on the above extracts using Merck Kiselgel thin layer plates as described by Stahl (1967). Lipids from both experimental and control extracts were applied in volumes containing three concentrations of total lipids, approximately 50, 100, and 200 μg. The solvent system was petroleum ether (bp 60–80°C): ether: acetic acid (70:30:1 v/v). The standards were contained in a TLC reference standard obtained from NU-CHECK Preparations, Elysian, Minnesota, U. S. A. Lipids were visualized with I_2 vapor and spraying with chromic sulfuric acid.

Since TLC showed 1,2-diglycerides to be the major glycerol-containing lipids in the above samples, diglycerides were quantified by the method of Wahlefeld (1974) after lipase hydrolysis.

Some of the data were analyzed by Student's *t*-test; in this report the term significant refers to statistical significance in this test at the 5% level or better. Most of the data is presented as mean ± the standard error of the mean (s.e.m.).

RESULTS

Preliminary experiments

In several initial experiments the *Leander* assay was used to quantify RPCA in heads, brain-CC-corpora allata complexes, and CC of several available insect species. Although all examined species contained some RPCA (see Table 1), variations between species amounted to about four orders of magnitude (*i.e.,* 0.1 to 920 *Leander* units/organ). Two locusts, *S. gregaria* and *L. migratoria,* exhibited the highest RPCA levels, while the lepidopteran *D. plexippus* and the dipteran *C. erythrocephala* contained the least RPCA.

The high level of RPCA in *S. gregaria* CC prompted further preliminary studies on this species. These assays clearly demonstrated RPCA in both CC and brains from adults of variable age and both sexes. In addition to testing these initial extracts in the *Leander* assay, the effects of *S. gregaria* CC extracts on the dark chromatophores of the *C. vulgaris* body, uropods, and telson, and on *C. maenas* erythrophores were also examined. Several assays on each crustacean species clearly demonstrated that *S. gregaria* CC extracts produced dark chromatophore pigment concentration in *C. vulgaris* and erythrophore pigment concentration in *C. maenas.* Comparable experiments dealing with the effects of synthetic RPCH in these two crustaceans (Herman and Josefsson, unpublished) demonstrated that the effect of RPCH was qualitatively identical in both species to that of *S. gregaria* CC extracts. On the basis of these early experiments, and the availability of

TABLE I

RPCA content of various insect species (CA represents corpora allata; CC, corpora cardiaca). All assays were conducted once on extracts of pooled tissues.

Species tested	*Leander* units/organ
Schistocerca gregaria	920/CC
Locusta migratoria	340/CC
Dytiscus sp.	120/CC
Tachycines asynamorus	28/CC
Tenebrio molitor	13/Head
Periplaneta americana	2/CC
Danaus plexippus	0.8/Brain + CC + CA
Calliphora erythrocephala	0.1/Head

reasonable numbers of this well-studied and convenient laboratory animal, *S. gregaria* was chosen for further experimental studies of insect RPCA.

RPCA during the S. gregaria *life cycle*

In subsequent experiments *Leander* assays of the RPCA present in extracts of whole heads of first, second and third instars, brains and CC of fourth instars, and brains, CC, and hemolymph of selected stages of the last instar and adults of both sexes were conducted. Single assays of pooled samples indicated that first, second, and third instar heads contained, respectively, 8, 16, and 310 *Leander* units/head, and that the fourth instar brain and CC contained 0.1 and 250 *Leander* units/organ, respectively. The results obtained from the last two developmental stages are summarized in Table II.

Several points emerge from the consideration of the above data and those of Table II. First, RPCA is present in all post-embryonic life cycle stages. There appears to be a rapid increase in activity during the first three instars (to about 40 times the initial value), followed by a slower increase into the adult stage. The mean combined adult CC RPCA value is about 100 times that found in the first instar, and mean combined adult CC and brain RPCA levels are significantly higher than those found in the fifth instar. Secondly, RPCA is present in both

TABLE II

RPCA content of Schistocerca gregaria *hemolymph, corpora cardiaca, and brains. Data are presented in* Leander *units/organ or per ml hemolymph, with the number in parentheses indicating the number of assays performed on separate pooled samples.*

Stage and sex	Hemolymph	Corpora cardiaca	Brains
Fifth Instar			
Male	—	460 ± 141 (3)	0.4 ± 0.1 (3)
Female	—	337 ± 77 (3)	0.3 ± 0.1 (3)
Combined	1.6 (1)	399 ± 84	0.3 ± 0.1
Adult			
Male	—	827 ± 205 (3)	1.9 ± 0.3 (3)
Female	—	797 ± 106 (3)	0.7 ± 0.3 (3)
Combined	1.0 ± 0.5 (3)	812 ± 115	1.3 ± 0.3

the brain and CC of at least the last three developmental stages. Thirdly, RPCA is present in comparable amounts in the brain and CC of both sexes; the data suggested the possibility of higher RPCA levels in males, but significant sexual differences were not observed. Fourthly, the individual assays showed a range of 440 to 2700 times as much RPCA in the CC as in the brain; RPCA is clearly concentrated in the former organ. Fifthly, RPCA is present in hemolymph from both the final instar and the adult stage. Finally, analysis of data obtained from specific stages of the last instar and the adult stage indicated significant alterations in CC RPCA content during each stage. In the last instar, mean values were 213 ± 19 (N = 2) and 491 ± 97 (N = 4) for, respectively, postmolt and intermolt plus premolt. By contrast, the adult mean value on the day of eclosion was 1120 ± 57 (N = 2), while the mean for 10 and 20 days post-eclosion was 658 ± 106 (N = 4). It therefore appears that CC RPCA increases during the fifth instar and decreases after adult fledging. In adults, the post fledging CC RPCA content decrease at 10 and 20 days was associated with an apparent increase in hemolymph RPCA activity (0.6 *vs.* 1.3 *Leander* units/ml, respectively).

The concentration of RPCA in the CC, the presence of RPCA in the hemolymph, and the apparent variations of RPCA during the life cycle, jointly suggested that RPCA might act as a neurosecretory hormone in *S. gregaria.* Fortunately, several reasonably convenient assays for such hormones in this species had been established by previous research (Goldsworthy and Mordue, 1974). The effects of synthetic RPCH in three of the test systems available were, therefore, examined. Using the techniques described by Mordue and Goldsworthy (1969), the effect of variable amounts of RPCH on heart rate and amaranth excretion were examined. Several attempts to demonstrate an effect of the synthetic hormone in these assays were unsuccessful. In certain experiments minor increases in heart rate and amaranth clearance were obtained, but the results were not significantly different from control values. In view of the relatively striking results with RPCH described below, further experiments with these two assays were not attempted.

RPCH effects on S. gregaria *hemolymph lipids*

Effect of RPCH on adults and immature animals. Initial experiments on hemolymph lipids examined the response of intact adults to injections of synthetic RPCH or *S. gregaria* CC extracts. The results of two separate experiments, using intact adults of both sexes and variable age, showed that both synthetic RPCH and CC extracts caused pronounced elevation of adult hemolymph total lipid levels. The values (in g total lipid/100 ml hemolymph) obtained one hr after injection were: adults injected with 1 μg RPCH in 50 μl distilled water, 4.23 ± 0.33 (N = 7); adults injected with an extract containing the equivalent of one CC in 50 μl distilled water, 4.62 ± 0.28 (N = 7); and adults injected with 50 μl distilled water, 1.17 ± 0.1 (N = 10). Comparable results were obtained in one experiment using neck-ligatured adults. Clearly, RPCH was adipokinetic in the presence or absence of the *S. gregaria* head. In numerous additional experiments, using *S. gregaria* from three separate colonies, elevation of hemolymph lipid was always obtained in response to suitable doses of RPCH or *S. gregaria* CC extracts in either sex or any age of the adult stage.

In two additional experiments the response of intact, mixed sex, intermolt fifth instars to injections of synthetic RPCH were examined. In the first experiment, animals were injected with hormone, and hemolymph was withdrawn for analysis after 60 min. An apparent response occurred in these animals, since five injected with RPCH, and five injected with distilled water, had total hemolymph levels of 0.47 ± 0.09 and 0.31 ± 0.04 g/100 ml, respectively. Since these results were not significantly different, the experiment was repeated with hemolymph samples taken both before injection and one hr after injection. When the results of this second experiment were calculated on the basis of increase/animal, 0.1 μg RPCH/locust ($N = 7$) caused an increase in hemolymph total lipid of 0.23 ± 0.04 g/100 ml, while distilled water ($N = 7$) led to an increase of 0.10 ± 0.02 g/100 ml. These latter results, although certainly less striking than those obtained with adults, were significantly different; it therefore appears that RPCH is adipokinetic in both immature and adult *S. gregaria.*

Dose-response curve after RPCH injections into intact adults. A further series of experiments examined the dose-response relationship between injected RPCH and adult hemolymph total lipid levels. In these experiments, adults of mixed sexes were injected with synthetic hormone, and the response was measured 1 hr after injection. The results of these studies (see Fig. 1) demonstrated that significant increases are obtainable with about 6 ng synthetic RPCH/animal, and that maximal responses require about 50 ng.

Effects of RPCH analogues in intact adults. In further experiments the effects of synthetic RPCH analogues on hemolymph total lipid levels in intact *S. gregaria*

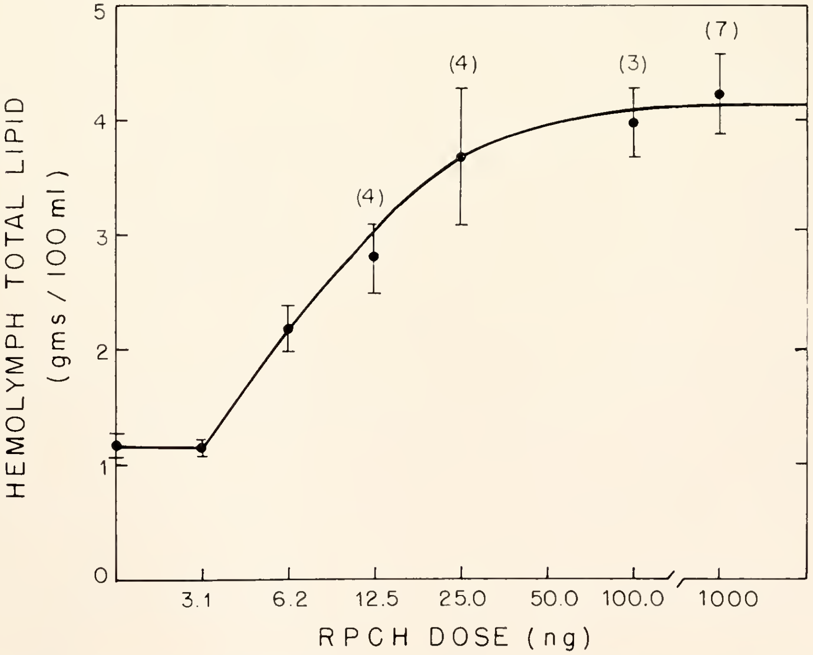

FIGURE 1. Dose-response curve of adipokinetic response of *Schistocerca gregaria* adults injected with synthetic RPCH with N in parentheses.

TABLE III

Effects of synthetic RPCH analogues on adult Schistocerca gregaria *hemolymph lipid. Data are presented as mean ± s.e.m. with N in parentheses. All peptides were injected at a dose of 1 μg/animal, and response was measured one hr after injection. RPCH is the first peptide in the table.*

Peptide tested	Total hemolymph lipid (gm/100 ml)
pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2 (6)	7.69 ± 1.20
pGlu-Leu-Asn-Tyr-Ser-Pro-Gly-Trp-NH_2 (6)	7.32 ± 0.88
pGlu-Pro-Gly-Try-NH_2 (6)	2.43 ± 0.30
pGlu-Leu-Asn-Phe-Ser-Pro-Gly-NH_2 (5)	2.18 ± 0.97
pGlu-Leu-Asn-Phe-Ser-Pro-Gly-OH (5)	1.66 ± 0.27
Distilled Water (6)	1.85 ± 0.29

adults were examined. The results of these studies, summarized in Table III, show that the tyrosine analogue, which has high activity in the *Leander* assay (Christensen *et al.,* in preparation) is apparently equally as effective as RPCH in *S. gregaria.* By contrast the tetrapeptide, which has low RPCH activity (Christensen *et al.,* in preparation), did not result in significant increases, but may have slightly elevated total lipid levels. The heptapeptide and heptapeptide amide, which are inactive in the *Leander* assay, were also without effect in *S. gregaria.* The adipokinetic effect of RPCH is specific to RPCH or structurally similar molecules.

RPCH effects on specific hemolymph lipids. After completion of one of the experiments on intact animals described above, 100 μl of hemolymph from RPCH-injected animals and 100 μl of hemolymph from control animals were collected and subjected to chloroform:methanol extraction, gravimetric determination of total lipid, and TLC analysis.

The results of the gravimetric analysis were comparable to those obtained by the colorimetric method (5.31 *vs.* 4.23 g/100 ml, respectively, for RPCH injected adults and 1.27 *vs.* 1.17 g/ml for controls). The higher value obtained by gravimetric analysis of hemolymph from RPCH-injected animals may be due to an absence of linearity in the colorimetric method at lipid levels over 4.0 g/100 ml (as reported by the manufacturer), or to the fact that the animals injected with RPCH had a somewhat longer time to respond to the injected hormone.

The TLC analysis indicated a substantial increase in hemolymph 1.2-diglyceride, and a possible decrease in both phospholipid and cholesterol in response to RPCH, but revealed no striking changes in other hemolymph lipid classes. Previous research (Mayer and Candy, 1969) had suggested a selective elevation of locust hemolymph 1.2-diglyceride in response to intrinsic adipokinetic hormone. We therefore decided to extend our TLC results by quantification of 1.2-diglyceride levels in hemolymph from both experimental and control animals. On the basis of an average molecular weight of 620 (diolein), the amount of 1.2-diglyceride was found to be 4.20 and 0.33 g/ml hemolymph, respectively, in RPCH-injected and control animals. Based on the above gravimetric analysis of total lipids, 1.2-diglycerides constituted 70% of the total hemolymph lipid in RPCH-injected animals, but only 26% of the total in control animals. These quantitative data, when coupled with our TLC results, clearly demonstrated that 1.2-diglycerides were the major lipids accumulating in *S. gregaria* hemolymph in response to RPCH injections.

RPCH and adipokinetic hormone localization in the neuroendocrine system.

Earlier research had reported the absence of locust adipokinetic hormone in brain (Mayer and Candy, 1969) and the concentration of adipokinetic hormone in the CC glandular lobe (Goldsworthy *et al.*, 1972). In further studies the *Leander* assay was used to quantify RPCA in the pars intercerebralis, brain minus pars intercerebralis, and CC glandular and storage lobes. The data from two such experiments are presented in Table IV.

As the above data show, RPCA was present in all regions examined, with 93% of the total brain RPCA localized outside the pars intercerebralis and 97% of the CC RPCA present in the glandular lobe. In one experiment, RPCA was also found in the corpora allata (0.06 *Leander* units/gland) and in the hypocerebral ganglion (26 *Leander* units/ganglion) of mature adults of mixed sexes. [We believe, in agreement with Thomsen (1943), that the RPCA in the hypocerebral ganglion may be due to the difficulty of separating it completely from the CC. By contrast, the corpora allata, which can be readily separated in this species, apparently contain a low level of RPCA.] The RPCA present in the brain in these experiments was considerably higher than that found in earlier studies (see Table II). We cannot account for this increase with certainty, but it may indicate that extracts of 10 brains/ml (instead of the 2 brains/ml used in Table II) yield more precise results.

In other experiments with *S. gregaria*, adipokinetic activity was assayed in CC glandular and storage lobe extracts. The results of these experiments (see Table V) generally agreed with those reported earlier (Goldsworthy *et al.*, 1972). Significant responses were observed with injections containing the equivalent of 0.4 lobes of each region, but only the glandular lobe extracts produced a significant response at 0.04 lobes/injection. Glandular lobes, therefore, contain at least ten times the adipokinetic activity of storage lobes. Very old mature males and females were used for these experiments; this may account for the high control values.

Absence of RPCH effect on L. adspersus *hemolymph lipids*

The above results suggested that RPCH might also influence hemolymph lipid levels in the shrimp *L. adspersus*. Experiments to test this possibility revealed that total hemolymph lipid levels (in g/100 ml) were: in intact shrimp, 1.94 ± 0.31 (N = 14); in eyestalkless shrimp, 1.24 ± 0.35 (N = 10); in eyestalkless shrimp

Table IV

RPCA localization in adult Schistocerca gregaria *brain and corpora cardiaca.* A and B represent the first and second experiments; pooled tissues are from male and female adults of both sexes used for extracts; and experimental duration = 1 hr.

Organ and region	*Leander* units/region			Mean % of total units
	A	B	Mean	
Brain				
Pars intercerebralis	0.4	0.3	0.4	7
Brain minus pars intercerebralis	4.2	4.4	4.3	93
Corpora cardiaca				
Glandular lobe	760	1350	1055	97
Storage lobe	50	8	29	3

TABLE V

Effect of glandular and storage lobe extracts on adult Schistocerca gregaria *hemolymph total lipids.* Data are presented as mean ± s.e.m. with N in parentheses; controls injected with 50 μl distilled water; and experimental duration = 1 hr.

Amount injected	Total hemolymph lipid (g/100 ml)		
	Controls	Storage lobe	Glandular lobe
0.4 lobes	3.86 ± 0.08 (8)	6.44 ± 0.63 (11)	6.16 ± 0.44 (11)
0.04 lobes	3.73 ± 1.07 (5)	4.73 ± 0.62 (6)	7.40 ± 0.93 (6)

injected with 50 μl 1.6% NaCl, 1.20 ± 0.22 (N = 11); and in eyestalkless shrimp injected with 1 μg RPCH in 50 μl 1.6% NaCl, 1.48 ± 0.21 (N = 14). The data did not therefore indicate an adipokinetic action of RPCH in *L. adspersus.*

DISCUSSION

The results confirm earlier reports of RPCA in representatives of the Orthoptera, Dictyoptera, Coleoptera, Lepidoptera, and Diptera, and add one new species (*D. plexippus*) to the list of insects possessing such activity. These data provide no information as to the absolute amount, or chemical nature, of the RPCA found in these insects. Future studies must determine if the observed species' differences in RPCA levels are due to variable quantities of the same substance or differing specific activities of a variety of substances.

These studies, and some others (Brown and Meglitsch, 1940; Thomsen, 1943), suggest that RPCA could be ubiquitous in insects. However, Hanström (1940) failed to demonstrate RPCA in whole head extracts of several insects, including all examined Thysanura, Dermaptera, Tricoptera, and Hymenoptera, and certain species of Odonata and Lepidoptera. Apparently, RPCA is not uniformly present in insects. The existing data do indicate that the highest RPCA levels routinely occur in Orthoptera. In these studies and those of Hanström (1940) and Thomsen (1943), a total of ten orthopteran species all produced strong responses in RPCH assays.

We have demonstrated RPCA in the cephalic neuroendocrine organs and hemolymph of the last two developmental stages of *S. gregaria* and have shown that significant variations apparently occur in CC RPCA content in the same stages (see Table II). In addition, RPCA can be readily demonstrated in extracts of heads or neuroendocrine organs in earlier stages of the life cycle, and RPCH seems to be adipokinetic in both immature and mature individuals. If RPCA resides in the *S. gregaria* adipokinetic hormone (see below), these findings imply that the adipokinetic endocrine system is present and functional in all stages of the *S. gregaria* life cycle. The following data provide strong evidence that *S. gregaria* RPCA is a substance, closely resembling shrimp RPCH, that functions as the adipokinetic hormone of this locust: first, synthetic RPCH is clearly adipokinetic in both intact and neck-ligatured locusts, and doses of about 6 ng are effective in promoting a significant response (see Fig. 1); secondly, the effect of RPCH is apparently limited to structurally similar peptides, such as the tyrosine analogue, and peptides with little or no RPCH activity in shrimp have little or no adipokinetic activity in *S. gregaria* (see

Table III); thirdly, synthetic RPCH acts in a manner at present indistinguishable from the adipokinetic hormone, *i.e.,* it selectively elevates hemolymph 1.2-glycerides (see Results and Spencer and Candy, 1976); fourthly, both RPCA and the adipokinetic hormone are concentrated in the CC glandular lobe (see Tables IV and V); fifthly, both RPCA and the adipokinetic hormone are apparently present in *S. gregaria, L. migratoria, P. americana,* and *T. molitor* (see Table I; Goldsworthy *et al.,* 1972; Stone *et al.,* 1976), and the available quantitative data agrees that *S. gregaria* contains more of both substances than *L. migratoria,* which in turn contains more of both substances than *P. americana.* In addition, after the completion of this research, two reports appeared dealing with the relationship of RPCH and the locust adipokinetic hormone. One paper presented evidence that injection of crustacean eyestalk extracts elevated locust hemolymph lipid levels, while pure locust adipokinetic hormone produced RPCH-like effects on the chromatophores of several crustaceans (Mordue and Stone, 1976). The other contained a proposed structure for locust adipokinetic hormone, indicating that RPCH and the adipokinetic hormone contain the same six amino acids in identical positions (Stone *et al.,* 1976). In view of the available information, the only reasonable conclusion seems to be that locust adipokinetic hormone and crustacean RPCH are biologically and structurally similar molecules.

It is of interest to compare the quantitative aspects of this study with those of Stone and her colleagues (1976). We found RPCA equal to about 50 ng RPCH/adult CC (mean value of 812 *Leander* units/adult CC in Table II, times 0.62×10^{-4} μg RPCH/*Leander* unit). In addition, the lowest effective dose in our adipokinetic assay was about 6 ng, and the dose required for maximal elevation of hemolymph lipid was about 50 ng (see Fig. 1). By contrast, Stone and coworkers reported 500–800 ng pure adipokinetic hormone/adult CC (glandular lobe only), a minimum effective dose of about 1 ng, and a dose of about 12 ng to be required for a maximal response. Adipokinetic hormone, therefore, appears to have some 6–10% of the activity of RPCH in the *Leander* assay, while RPCH is about 20% as effective as pure adipokinetic hormone when assayed on *S. gregaria.*

We have demonstrated significant RPCA in *S. gregaria* brains, but previous workers failed to find evidence for a locust brain adipokinetic hormone (Mayer and Candy, 1969). Our experiments suggest that failure to demonstrate brain adipokinetic hormone may be explained on the basis of assay sensitivity. The *S. gregaria* adult brains assayed (see Table II) contained a mean value of 1.3 *Leander* units/brain. This amount of RPCH (about 0.08 mg) is about 1% of that required per animal for a significant response in our adipokinetic assay (see Fig. 1), but it is considerably higher than that used for the lowest standard (4 pg/shrimp) in the *Leander* assay. If, as discussed above, pure adipokinetic hormone is equivalent to 10–15 times the RPCA measured in the *Leander* assay, this would mean that each brain contains no more than 1–2 ng pure adipokinetic hormone. Since previous studies (Mayer and Candy, 1969) apparently injected the equivalent of 0.1 brain/locust, and a significant response in the adipokinetic assay requires 1–2 ng pure adipokinetic hormone (Stone *et al.,* 1976), it is not surprising that brain adipokinetic hormone activity was not observed. (A similar conclusion is reached if the higher values for brain RPCA of Table IV are used.)

The data (Table IV) suggest that RPCA is manufactured in the pars intercerebralis, accumulates in some other region of the brain, and is eventually transported to the CC storage lobe. This interpretation would account for the low pars intercerebralis RPCA, and the higher RPCA levels in the remainder of the brain and in the storage lobe. The presence of RPCA in all brains assayed indicates that storage lobe RPCA comes from the brain; its presence in storage lobe extracts is probably not due to attached portions of the glandular lobe. In addition, RPCA certainly accumulates in the CC glandular lobe. It may be that the glandular lobe RPCA originates in the brain or storage lobe; the histology of the *S. gregaria* CC is compatible with this possibility (Highnam, 1961). An alternative explanation would be the existence of two RPCA substances, one produced by the pars intercerebralis and the other by the glandular lobe intrinsic cells. The significance of the presence of RPCA in the *S. gregaria* CA remains to be determined; Thomsen (1943) found low RPCA activity in the CA of several species.

The chromatophorotropic function of RPCH, the hormone-like action of RPCH in *S. gregaria,* the presence of RPCA in a large number of different arthropods, and the apparent structural similarity between RPCH and the locust adipokinetic hormone, jointly suggest that several activities attributed to arthropod neurosecretory hormones could be mediated by a family of RPCH-like peptides. It will therefore be of great interest to characterize and synthesize RPCH-like peptides from different sources, and to investigate the hormonal action of these substances in a variety of arthropod species. Such data could lead to major advances in understanding the evolution of arthropod (or invertebrate) hormones, specifically, and the evolution of peptide hormones generally. It would also provide insight into the feasibility of using substances with RPCH-like activity, or chemicals interfering with such activity, for purposes of arthropod regulation. Patently, if RPCH-like peptides act hormonally in a diversity of arthropods, attempts to regulate arthropod populations by manipulations of systems mediated by RPCH-like molecules would require thoughtful consideration.

We are extremely grateful to those individuals who provided one or more of the species used in this study: Professors S. O. Anderson, E. Thomsen, L. Hagerman, S. Tuxen, S. Rasmussen, and P. Cherubino were all particularly helpful. Special thanks are due to the Director and staff of the Centre for Overseas Pest Research, especially Dr. R. Roome, for providing W. S. Herman with animals, facilities, and warm hospitality during a collecting trip. We additionally appreciate the very able technical assistance of Ms. B. Harder and Ms. V. Strange-Peterson. J. I. Ardery and W. S. Weidel also made unique contributions to this research.

SUMMARY

1. Red pigment concentrating hormone-like activity (RPCA) has been demonstrated and quantified in the head or cephalic neuroendocrine system of several insects. *Schistocerca gregaria* adults contained the highest levels of RPCA.

2. RPCA was found in all post-embryonic life cycle stages of *S. gregaria,* and seems to be present in equal quantities in both males and females. Adult locusts contain significantly more RPCA than do immature animals.

3. RPCA is concentrated in the locust CC glandular lobe but is also present in the CC storage lobe, brain and hemolymph of both mature and immature animals.

Significant variations in CC RPCA content have been demonstrated in both mature and immature stages.

4. Synthetic red pigment concentrating hormone (RPCH) is adipokinetic in both intact and neck-ligatured *S. gregaria* adults and in intact immature animals. Doses of about 6 ng and 50 ng, respectively, result in minimal and maximal adipokinetic responses. RPCH selectively elevates hemolymph 1,2-diglycerides.

5. RPCH in large doses was without effect on crustacean hemolymph lipid levels.

6. Evidence favoring the biological and structural similarity of RPCH and locust adipokinetic hormone is discussed.

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DESCRIPTION OF A NEW SPECIES, *CRANGON HANDI*, AND NEW GENUS, *LISSOCRANGON*, OF CRANGONID SHRIMPS (CRUSTACEA: CARIDEA) FROM THE CALIFORNIA COAST, WITH NOTES ON ADAPTATION IN BODY SHAPE AND COLORATION

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Shrimps of the genus *Crangon* occurring in shallow waters of the Californian coast have been known taxonomically for 75 to 120 years. Although they are an abundant and important element of the inshore epibenthic fauna, contribute to a minor fishery in San Francisco Bay (Israel, 1936; Skinner, 1962; personal observations), and are common in museum collections, they have been infrequently studied. In the only major studies since Schmitt's (1921) taxonomic monograph of the decapod Crustacea of California, Israel (1936) detailed the growth, reproduction, and fisheries statistics for *Crangon franciscorum* and *C. nigricauda* in San Francisco Bay; and Krygier and Horton (1975) considered growth, reproduction, and local distribution for the same species in Yaquina Bay, Oregon.

A distinctive species of *Crangon* from Californian nearshore waters, *Crangon handi* n. sp. is described in this study. It first came to our attention in Horseshoe Cove, Bodega Head, Sonoma County, California, where it is remarkably camouflaged on coarse-grained, sandy substrates. In order to effect proper subgeneric placement, Zarenkov's (1965) *Crangon* subgenera are evaluated and a new genus is proposed. Adaptation in body shape and color pattern among *Crangon* of the central California coast are further discussed.

Four related species of the genus *Crangon* have long been known from shallow waters of the central California coast: *C. nigricauda* Stimpson, 1856, *C. franciscorum* Stimpson, 1856, *C. nigromaculata* Lockington, 1877, and *C. alaskensis* Lockington, 1877 (= *C. alaskensis elongata* Rathbun, 1902). These species, along with *Crangon handi*, have carapace spination limited to one median gastric spine and one pair of hepatic spines and further differ from other Pacific coast *Crangon* by having a short unornamented rostrum and a sixth abdominal segment lacking a pair of prominent dorsal keels (carinae) but having a sulcate ventral surface. These are termed the *smooth, sulcate species group*. *"Crangon" stylirostris* Holmes, 1900, is also included in the analyses and discussion, as it is ecologically and geographically sympatric with species of the smooth, sulcate group. Other Pacific coast *Crangon* have a convex ventral surface on the sixth abdominal segment, possess dorsal abdominal carinae, or have additional carapace spines.

MATERIALS AND METHODS

Although the first specimen from Horseshoe Cove was captured in 1965, we were unable to obtain more than a few additional specimens until 1975, when the first specimen from Shell Beach, Sonoma Coast State Beach, north of Bodega Head was collected from a low intertidal tidepool. Specimens can now be collected at will from this locality with a long-handled dipnet.

All measurements were done with a vernier caliper to 0.1 mm. Carapace length was measured from the posterior margin of the orbit to the dorsal midline of the posterior margin of the carapace. Total length was taken from the anterior tip of the spine of the antennal scale to the posterior margin of the uropods with the shrimp extended flat on its dorsal surface. Repeated readings within 0.1 mm showed this measurement to be a very precise estimator of overall length. The curve of the body and the presence of eggs on ovigerous females do not permit comparable precision for measurements of the usual "tip of rostrum to tip of telson" total length dimension of shrimp systematics. Israel (1936) reported 6–8% variation from tip of rostrum to tip of telson depending on how the specimen was held. Thus, the measurement inclusive of antennal scales and uropods is used for the morphometric studies, while the usual tip of rostrum to tip of telson measurement is given in the species description of *C. handi* for comparative purposes. Length of the antennal scale was measured along the lateral margin from its basal articulation to the tip of the spine. Antennal scale width was measured at the widest point and included the spine. Chela length was measured along the lateral margin of the propodus. Chela width was taken at the widest point. Length of the sixth abdominal segment was measured along the dorsal midline.

Relative growth is described here by the power function $y = \beta x^{\alpha}$, where x is the reference dimension, y is the dependent variable and β and α are constants. This curvilinear relationship becomes linear on logarithmic coordinates, such that $\log y = \alpha \log x + \log \beta$, where β represents the size of y when $x = 1$, α is the slope of the logarithmically transformed equation and describes the rate of growth of the dependent variable in comparison to the reference dimension. Allometric growth is positive if $\alpha > 1$, and negative if $\alpha < 1$. The growth relationship is isometric if $\alpha = 1$ (Teissier, 1960; Gould, 1966). Growth is considered isometric if $0.9 < \alpha < 1.1$. This is analogous to the definition of arithmetic growth (as opposed to progressive and regressive growth) used by Kurata (1962) and Hepper (1967).

Frequently, the greatest linear dimension (in this case total length) is chosen as the reference dimension. However, this measurement is notoriously variable in flexible animals such as shrimp. Carapace length is used here as the reference dimension, because it is the greatest measurement possible on a relatively rigid cuticle and relates to overall body size.

For color and background pattern adaptation, animals were placed in containers with either Shell Beach or Horseshoe Cove substrate for 14 days prior to photography. Sediment samples collected in mid-summer were analyzed, and mean grain size (based on graphical phi percentile measures) was determined following methods given by Folk (1968), involving sorting dry sand through a consecutive graded series of Tyler sieves using a mechanical shaker.

Specimens examined are on deposit at the National Museum of Natural History (NMNH), California Academy of Sciences (CAS), Allan Hancock Foundation, University of Southern California (AHF), Santa Barbara Museum of Natural History (SBMNH), and Bodega Marine Laboratory Synoptic Collection (BMLSC).

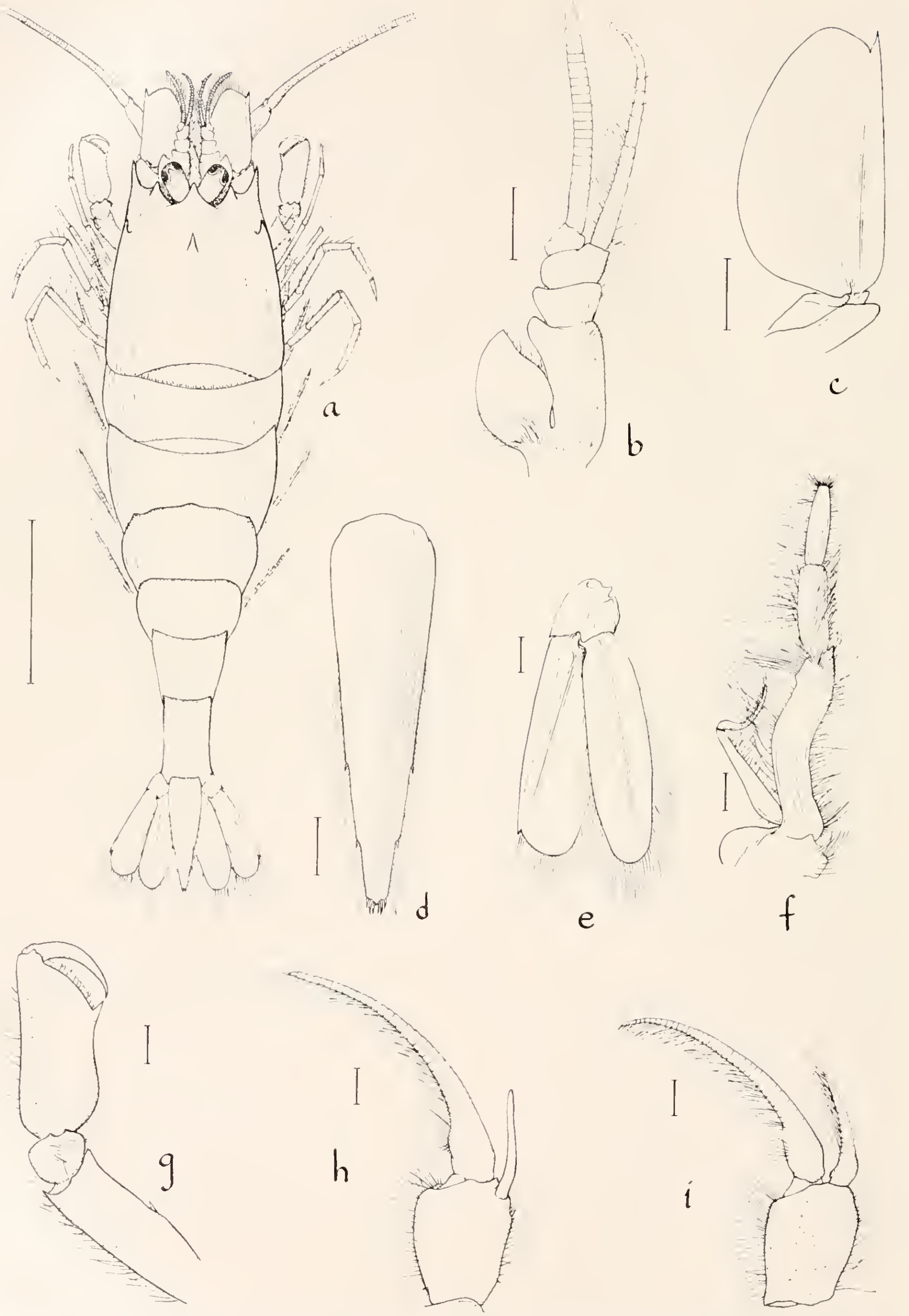

FIGURE 1. *Crangon handi*, new species: a, adult ovigerous female, total length 51 mm; b, antennule (first antenna) and antennular scale; c, antennal scale (scaphocerite); d, telson; e,

TAXONOMY

Crangon handi new species (Figure 1 a–i, Figure 2, a, d, g)

Crangon sp., Carlton and Kuris, 1975: 388, 404

Diagnosis

Antennal scale short, one-half length of carapace or less, and very broad (length less than or about equal to two times width), acicle not exceeding blade; flagella of first antenna equal in length; sixth abdominal segment very short, dorsum smooth; first pleopod lacking spine on anterodistal corner.

Description (*adult ovigerous female, Fig. 1a*)

Antennule (first antenna) (Fig. 1b) with flagella extending beyond antennal scale; flagella equal in length, the inner (lower) flagellum not as robust as outer; peduncle about one-half length of antennal scale, approximately one-half length of antennal peduncle. Antenna with peduncle about two-thirds length of antennal scale; antennal flagella about three-fifths body length. Antennal scale (scaphocerite) (Fig. 1c) short, one-half length of carapace or less, and broad, length generally less than two times width; spine (acicle) and anterointernal (anteromesial) margin of blade codistant. Eye stalk and ocellus reaching well beyond rostrum. Carapace with one medial gastric spine and one pair of hepatic spines. Rostrum short, straight, unornamented, grooved above, round at tip. Abdomen with segments typical, unornamented, except for very weak medial monocarination; abdominal segment very short, about two-thirds length of telson, smooth dorsally, sulcate ventrally but not deeply so. Telson (Fig. 1d) smooth dorsally with lateral spination typical: a single tiny spine on each margin about one-seventh the distance from tip and again about one-third the distance from tip; tip acute, flanked on each side by three tiny spines. Uropods (Fig. 1e) subequal, as long as telson, spine of exopod without setae or spines on margin. Maxilliped 3 (Fig. 1f) typical. Cheliped (Fig. 1g) with chela stout, dactyl turned down at approximately 45° angle; spine short, acute, margin of propodus lightly setose. Pereopods typical. Pleopods (first, Fig. 1h; second, Fig. 1i) normal, except that first pleopod lacks a spine on the anterodistal corner.

Size

Largest specimen examined (holotype), total length, 50.3 mm; carapace length, 10.7 mm (from Horseshoe Cove, Bodega Head). Range: male carapace length 6.1 mm; females, ovigerous, 10.2–10.7 mm, not ovigerous, 8.1–9.6 mm; small, unsexed, 3.2–3.8 mm.

Color in life (*adult female*)

The dorsal surface presents a complex pattern of almost a dozen colors. On the antennal scale are cloudy-white patches anteriorly and posteriorly, separated by a

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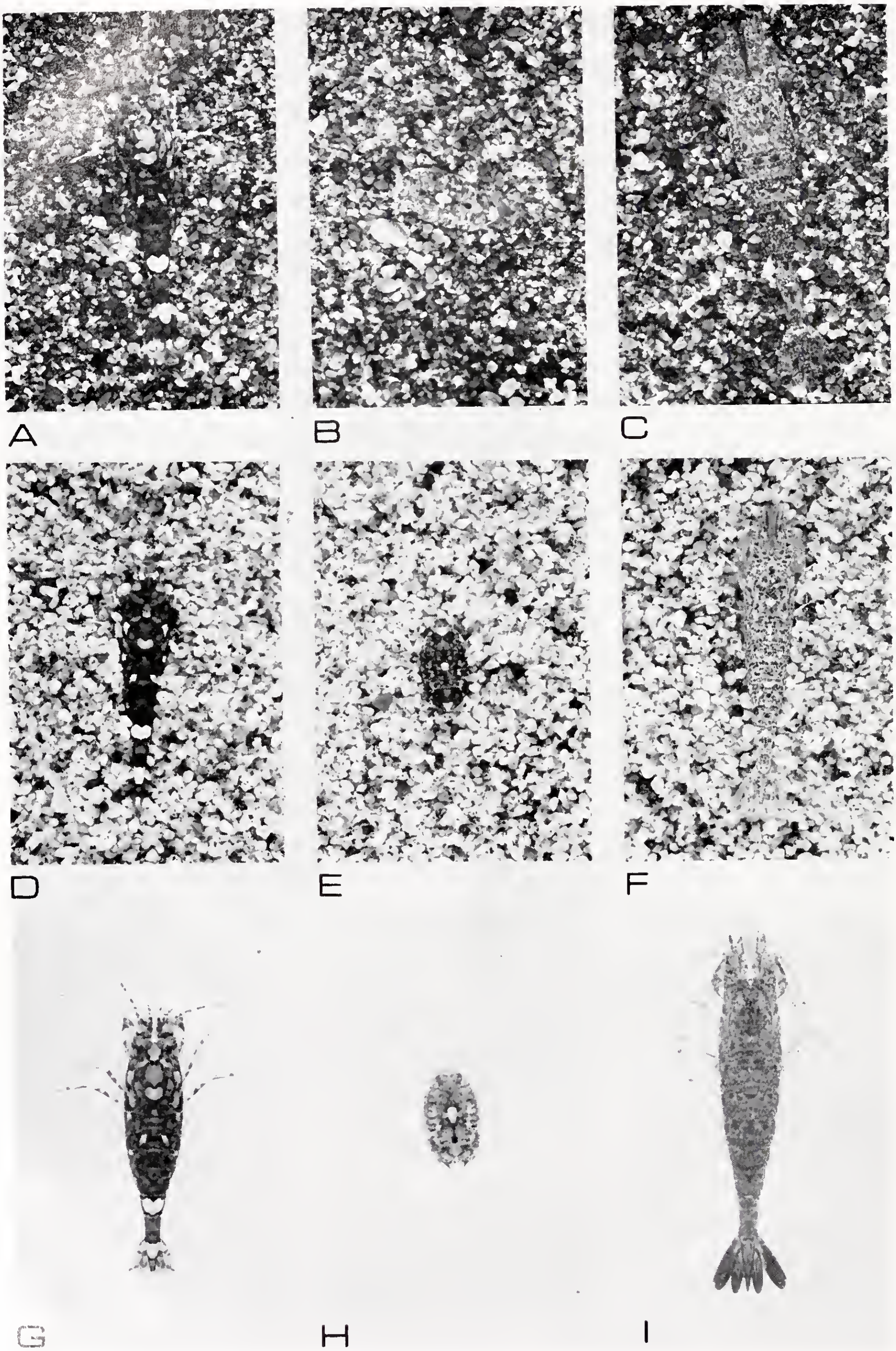
uropods; f, maxilliped 3; g, cheliped; h, pleopod 1; and i, pleopod 2. All are left, except for c. Scale on a = 1 cm; others = 1 mm.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black band; another black bar is at the base of the scale. The peduncle of the first antenna is brown, black, and white-marked; the flagella are green, brown and/or black-marked. The peduncle of the second antenna is black and orange, with the flagella black-and-green banded with alternating white areas. On the carapace there is an anterior irregular white patch, and on most specimens a small notch at the base of this patch which conforms to the tip of the median gastric spine. The rostrum is white, and may or may not be black-tipped. The remainer of the carapace pattern is complex, with regions of purple, black, brown, white, and orange. The gastric spine is brown, with a distal brown band below a white tip. Hepatic spines are translucent. The posterior margin of the carapace is marked by a subrectangular white patch.

Abdominal segments are generally translucent with brown, white, red, black, and green markings. Notable and generally consistent on the abdominal segments are two gently sloping white marks on either side of the medial line of the second segment; a brown-black dot on the posterior medial margin of the third segment, and a very conspicuous heart-shaped white patch, generally with a central black dot, on the fifth abdominal segment. The anterior half of the telson consists of an elongate pink-white patch; the central area of the telson is black with a pink-white patch; the posterior area of the telson is white. On the uropods distally and proximally are broad white bands, separated by brown and orange bars and markings. Abdominal coxae are brown with black and/or yellow marks.

The cheliped hand is distally black and proximally white, marked here and there with white and green markings. Remaining articles are black-spotted. Pleopods have brown markings, the lateral surface with a long yellow line or a line of yellow spots; pereopods are variously translucent, or with yellow, orange, and black spots. The carpus of pereopods 4 and 5 has a white line or a line of white spots medially.

Crangon handi presents, then, a highly disruptive pattern blending in superbly with its surrounding coarse sand substrate (Fig. 2a, d). On or near the medial dorsal line of the shrimp, there are a series of approximately equally-spaced conspicuous white marks: white patches on the antennal scale, white areas anteriorly and posteriorly on the carapace, two white marks on the second abdominal segment, a white heart-shaped patch on the fifth segment, and white patches anteriorly and posteriorly on the telson and uropods.

Etymology

We are pleased to name this species in honor of Dr. Cadet H. Hand, Jr., founder and Director of the Bodega Marine Laboratory and a keen student of the central California invertebrate fauna. Dr. Hand took an interest in this work and other projects, provided a model for sharp-eyed field work, and encouraged Robert Sikora's exploratory sampling of Horseshoe Cove.

Type locality and type material

Horseshoe Cove, Bodega Head, Sonoma County (38° 18′ 30″ N, 123° 4′ W). Holotype, adult ovigerous female (Fig. 1a), total length 50.3 mm, total carapace

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FIGURE 2. a, d, g: *Crangon handi*, new species; b, e, h: the isopod *Tecticeps convexus;* c, f: *Lissocrangon stylirostris;* i: *Crangon nigricauda;* a–c are on coarse substrate from Shell Beach, d–f are on coarse substrate from Horseshoe Cove, and g–i are on smooth white backgrounds.

length 10.7 mm, length from tip of rostrum to tip of telson 43.2 mm, collected 1965, R. Sikora, deposited at NMNH, no. 169870. Paratypes deposited at CAS, and AHF; additional material at NMNH, CAS, AHF, SBMNH and BMLSC.

Geographic range and bathymetry

Crangon handi occurs from the intertidal zone to 55 m from Shell Beach, Sonoma County, to Bahia Colnett, northern Baja California. North of Point Conception, collections are centered around Sonoma County (Shell Beach, Carmet, Horseshoe Cove), and Monterey Bay (Santa Cruz, Pacific Grove, Monterey), with one collection in San Luis Obispo County. In southern California several subtidal collections were from the Channel Islands.

Habitat

With the possible exception of a "shore" record at Corona del Mar, Orange County, all specimens south of Sonoma County have been dredged or lack detailed habitat notes.

All Sonoma County specimens of *C. handi* have been collected from beach wrack, tidepools or shallow water over a very coarse to coarse sand substrate typical of small beaches, coves, and surge channels along the Marin, Sonoma, and Mendocino County rocky coasts of California. Most of this coast lies east of the San Andreas Fault. Here, such as at Shell Beach, the sands are dark, consisting largely of chert, graywacke, greenstone and serpentine clasts derived from the Franciscan Formation which lies along the fault zone. West of the fault zone, beach sands may be light and granitic, such as the beach at Horseshoe Cove on Bodega Head (which also has a small amount of Franciscan sediments washed around Mussel Point on Bodega Head).

Sand size analyses from Horseshoe Cove and from Shell Beach show that these habitats have a much larger mean grain size (approximately 1.45 mm and 0.92 mm, respectively) than the sandy or muddy substrates over which other species of *Crangon* considered here normally occur (for example, Campbell Cove, Bodega Harbor, a medium-sand sandflat, with aeolian-deposited sediments derived largely from local sand dunes, has a mean grain size of 0.35 mm).

Co-occurring with *C. handi* in Sonoma County are cottid fish, polychaete worms, and the sphaeromatid isopod *Tecticeps convexus* Richardson. To the south, *C. handi* has been recorded regularly over sand, gravel, and "shell" bottoms with a number of associated species including *C. nigricauda, C. alaskensis, C. alba, C. holmesi,* the echinoid *Dendraster* and the brachyuran *Pugettia.*

Epizoics, food, and predators

Specimens of *C. handi* examined were almost always free of epibiotic organisms. A specimen of the foraminifer *Rosalina columbiensis* was noted on the left third maxilliped of an adult female from Shell Beach (July 11, 1975).

A single specimen of a small gammarid amphipod, *Hyale frequens* (Stout, 1913), along with numerous sand fragments, was found in the stomach of a *Crangon handi* collected at Shell Beach (July 10, 1975). A specimen of an oedicerotid gammaridean amphipod and sand particles were also found in the buccal region of a *C. handi* from beach drift on Horseshoe Cove (June 27, 1968).

Predators upon *C. handi* in the northern half of its range are unknown. However, five specimens were removed from the stomach of a thornback skate, *Platyrhinoides triseriata,* collected on Naples Reef, 11 km north of Santa Barbara, California, over a coarse sand substrate. The thornback skate occurs in shallow water to 46 meters from near Thurloe Head, Baja California to San Francisco (Miller and Lea, 1972).

Comparison with related species and morphometrics

A new key to the five *Crangon* species of Schmitt's (1921) Group I, A and B1, and to *C. handi* follows. Distinctions between two often misidentified species, *C. nigricauda* and *C. alaskensis,* are clarified.

1. No gastric spine; rostrum narrow, tip pointed, curving strongly downward; telson shorter than uropods; first (antepenultimate) article of third maxilliped broadly expanded .... *stylirostris*

— One gastric spine; rostrum relatively broad, tip round, straight; telson equal to or longer than uropods; first article of third maxilliped narrow, not dilated 2

2. Finger of hand (dactyl of chela) turned down almost parallel (180°) to hand; an acute spine on posterodorsal corner of fifth abdominal segment; inner flagellum of first antenna more than two times as long as outer flagellum .... *franciscorum*

— Finger of hand at a 45° angle, or less, to hand; no spine on posterodorsal corner of fifth abdominal segment; inner flagellum of first antenna distinctly less than two times as long as outer flagellum 3

3. Flagella of first antenna equal in length; length of antennal scale about equal to or less than two times width; spine of antennal scale not exceeding blade; anterodistal corner of first pleopod without a spine .... *handi*

— Inner flagellum of first antenna distinctly longer than outer flagellum; antennal scale length always greater than two times width; spine of antennal scale almost always distinctly exceeding blade (common exception is *nigricauda*); anterodistal corner of first pleopod with a spine 4

4. Tip of telson without three small spines flanking each side (but with a single small spine on each side slightly proximal to tip); dorsum of sixth abdominal segment smooth, without a distinct row of small setae; antennae as long or longer than body; finger of hand at about a 30° angle to hand [*in living specimens,* always distinguished by one prominent circular spot (blue center with a blue and then a yellow ring) on side of sixth abdominal segment; this often fading in preservative] . . . *nigromaculata*

— Tip of telson with three spines on each side; dorsum of sixth abdominal segment slightly grooved, with a distinct row of central setae (may be worn) (*living specimens* never with colored spot on sixth abdominal segment) 5

5. Antennal scale blade tip narrow, spine long, much exceeding blade; scale greater than two thirds length of carapace; finger of hand at about 45° angle to hand; antennae about two-thirds body length .... *alaskensis*

— Antennal scale blade tip broad, spine generally short, hardly exceeding blade; scale about two-thirds length of carapace; finger of hand tending toward transverse, at about 30° angle to hand; antennae from two-thirds body length to as long as body (a variable species) .... *nigricauda*

In Schmitt's key (1921, p. 82) *C. handi* keys as far as I-B-1-a-ii, but will not key to species. *Crangon handi* lacks the large circular spot on the much longer sixth abdominal segment of *C. nigromaculata,* which also possesses a much longer antennal scale. *Crangon handi* possesses only a very weakly monocarinate fifth abdominal segment; the "carina" is weaker than that of *C. nigricauda. Crangon handi* is further distinguished from *C. alaskensis* and *C. nigricauda* by the distinctly shorter and broader antennal scale of the former, and by the more elongate chelae of both species. *Crangon handi* is keyed in *Light's Manual* (Carlton and Kuris, 1975) as *Crangon* sp. In Kozloff (1974, p. 164) *C. handi* will key (13a) to *Crangon nigricauda,* but can be distinguished from that species by the characters noted above.

When log total length is compared with the reference dimension log carapace length (Fig. 3), *Crangon handi* is the most robust species, followed closely by "*C.*" *stylirostris. Crangon nigromaculata* and *C. alaskensis* are similar and most attenuate. *Crangon nigricauda* and *C. franciscorum* are similar and intermediate.

Relative length of log sixth abdominal segment, when compared to log carapace

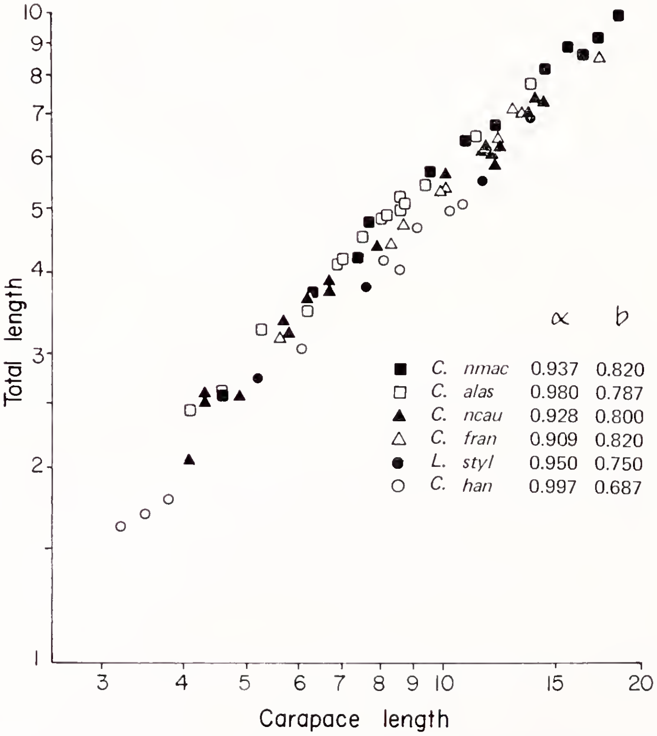

FIGURE 3. Relative growth of log total length compared with log carapace length for six crangonid shrimp; abbreviations and sample sizes (N) include *C. nigromaculata* = *C. nmac.,* N = 13; *C. alaskensis* = *C. alas.,* N = 15; *C. nigricauda* = *C. ncau.,* N = 19; *C. franciscorum* = *C. fran.,* N = 10; *L. stylirostris* = *L. styl.,* N = 5; and *C. handi* = *C. han.,* N = 9. Pearson product-moment correlation coefficients (r) were uniformly very high with a range of 0.980–0.999, for all groups in Figures 3–8.

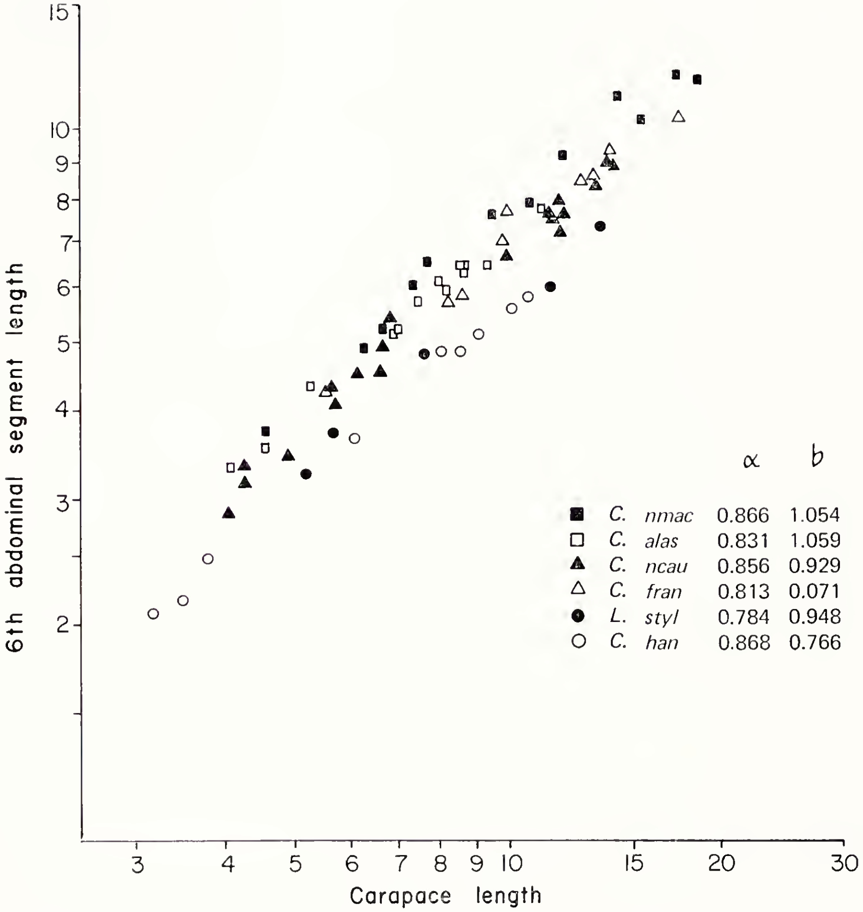

FIGURE 4. Relative growth of log sixth abdominal segment length compared with log carapace length for six crangonid species. See Figure 3 for key to species, abbreviations and sample sizes, except for *C. nigromaculata*, N = 12.

length (Fig. 4), varies between species more than does log total length *versus* log carapace length. *Crangon handi* has the shortest sixth abdominal segment, followed by *"C." stylirostris*. *Crangon nigricauda* and *C. franciscorum* are similar, while *C. nigromaculata* has the longest sixth abdominal segment.

Log chela length *versus* log carapace length shows the least variation between species (Fig. 5). *"Crangon" stylirostris* has the shortest chela; *C. franciscorum*, the longest. The remaining four species are similar and intermediate.

Log chela length *versus* log chela width shows more between-species variation than do the three allometric relationships considered above (Fig. 6). *Crangon franciscorum* has very narrow chelae. *Crangon nigromaculata* and *C. alaskensis* are similar and have the next most elongate chelae. *Crangon nigricauda* and *C. handi* are similar with rather wide chelae, while *"C." stylirostris* has the widest

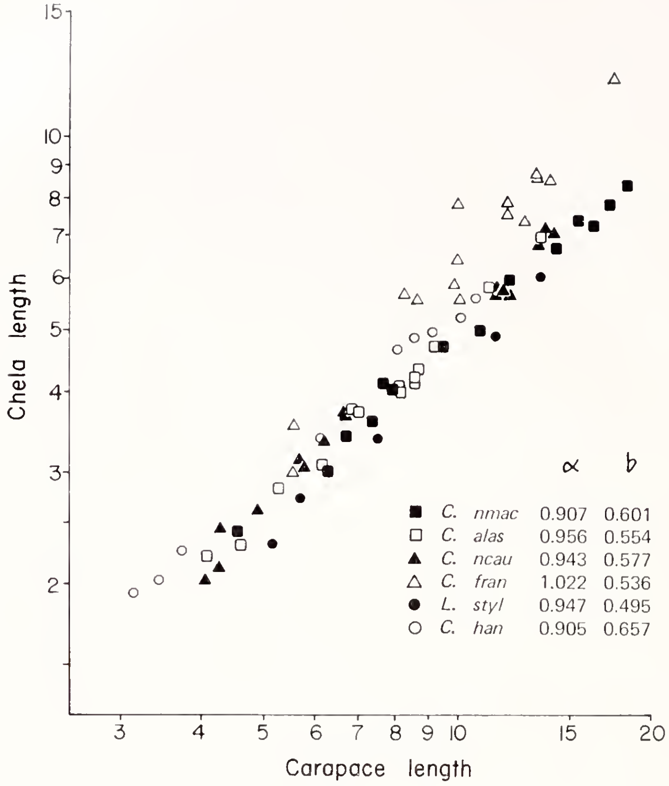

FIGURE 5. Relative growth of log chela length compared with log carapace length for six crangonid species. See Figure 3 for key to species, abbreviations and sample sizes, except for *C. alaskensis*, N = 14.

chela of these six species. The slope of the *C. franciscorum* regression is 0.768, the other five species have similar slopes, between 0.948 and 1.082.

Log antennal scale length *versus* log carapace length exhibits greater between-species variation than do the four preceding relationships (Fig. 7). Antennal scale length of *C. handi* is considerably shorter and has a lower slope than the other five species. *Crangon nigricauda* and *C. franciscorum* are similar and have the next shortest antennal scale length. *Crangon alaskensis* and *"C." stylirostris* have distinctly longer antennal scales compared to the three previous species. *Crangon nigromaculata* has a slightly longer antennal scale than the preceding two species, but the relationship between log antennal scale length and log carapace length may be curvilinear for *C. nigromaculata*. Large and small individuals of *C. nigromaculata* approximate the *C. alaskensis* and *"C." stylirostris* relationship.

Between species, variation in the log antennal length-width relationship is similar to the variation in the preceding relationship (Fig. 8). *Crangon handi* has much the widest antennal scales, showing no overlap with the species having the next widest antennal scale, *C. nigricauda*. The remaining four species have a similar antennal scale length-width relationship, all being distinctly narrower than *C. nigricauda*. *Crangon nigromaculata* appears to have a slightly curvilinear relationship with the largest specimens having relatively wide antennal scales.

Generic revisions

An attempt to assign *C. handi* to a proper subgenus, as defined by Zarenkov (1965), has led us to propose the following partial revision of crangonid taxa falling within Schmitt's (1921) group I, those species with the gastric region of the carapace not depressed. Species here appear to fall into three natural groups, not previously defined as such: a genus lacking gastric spines (*Lissocrangon*, new genus), a genus with one median gastric spine (*Crangon*) and a genus with two gastric spines (*Neocrangon*). These three genera may be distinguished by several features in addition to gastric spination.

Family Crangonidae Bate

Lissocrangon, new genus

Type species: *Crangon stylirostris* Holmes, 1900.

Generic diagnosis: No gastric spines on carapace. Rostrum long, narrow, tip pointed, sharply decurved. Telson shorter than uropods. Sixth abdominal segment without a pair of dorsal carinae.

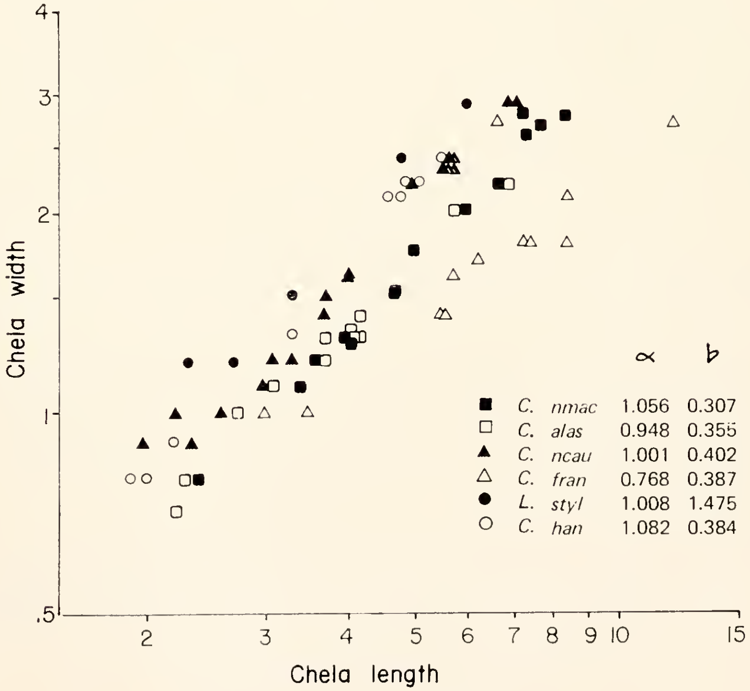

FIGURE 6. Relative growth of log chela width compared with log chela length for six crangonid species. See Figure 3 for key to species, abbreviations and sample sizes, except for *C. alaskensis*, N = 14.

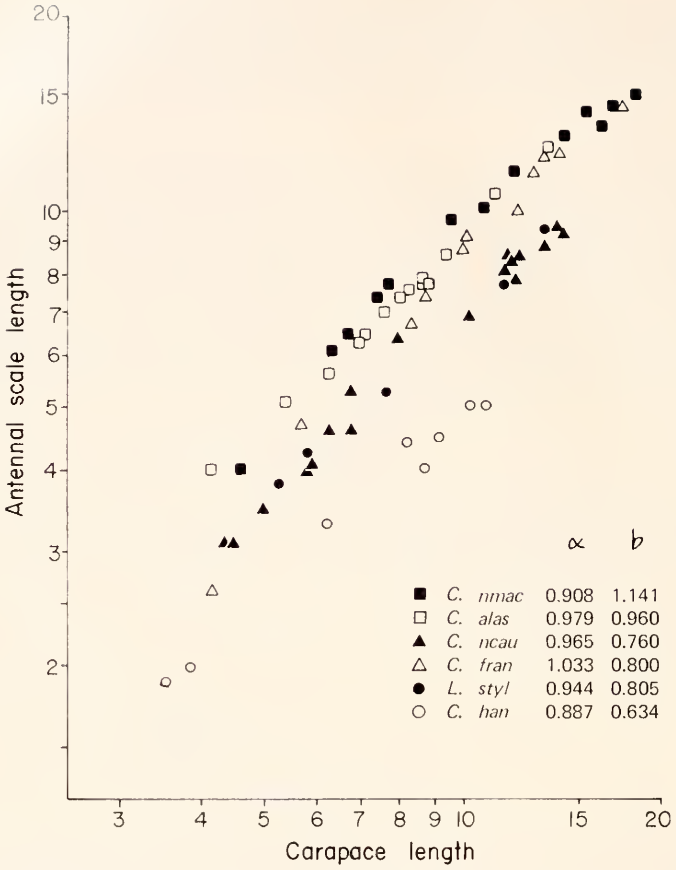

FIGURE 7. Relative growth of log antennal scale length compared with log carapace length for six crangonid species. See Figure 3 for key to species, abbreviations and sample sizes.

Etymology: From the Greek *lissos*, smooth, and *crangon*.

Remarks: Monotypic. Members of *Crangon* and *Neocrangon* possess a straight or upturned rostrum and a telson as long as or longer than the uropods, further separating them from this group. *Lissocrangon stylirostris* also possesses a broadly dilated first article of the third maxilliped, a character shared with *Crangon* (*Crangon*) *alba* Holmes, 1900.

Crangon Fabricius, 1798

Generic diagnosis: We here restrict *Crangon* to those shrimp with one medial gastric spine, straight or upturned rostrum, and a telson equal in length to or longer than the uropods. Other characters are those previously established for the genus. Two subgenera are distinguishable:

(*Crangon*) Fabricius, 1798

Type species: *Cancer crangon* Linnaeus, 1758.

Subgeneric diagnosis: Sixth abdominal segment smooth, lacking prominent dorsal carinae.

Remarks: Two groups of *Crangon* (*Crangon*) are apparent, a *sulcate group,* with the ventrum of the sixth abdominal segment sulcate (*C. nigricauda, C. franciscorum, C. nigromaculata, C. alaskensis, C. handi, C. crangon,* and *C. septemspinosa* Say, 1818, and perhaps *C. affinis* de Haan, 1849), and a *convex group,* with the ventrum of the sixth abdominal segment convex (*C. alba* and *C. holmesi* Rathbun, 1902). The anterolateral angle of the antennal scale blade, where it joins the antennal scale spine, is adnate in the convex group but not in the sulcate group. The convex species also do not appear to reach as large a body size as do species of the sulcate group.

(*Steiracrangon*) Kinahan, 1862, revived and amended

Type species: *Crangon allmanni* Kinahan, 1857.

Subgeneric diagnosis: Sixth abdominal segment with two prominent dorsal carinae.

Remarks: *Crangon dalli* Rathbun, 1902, *C. sagamiense* Balss, 1913, and *C. geniculata* Yokoya, 1933 are also included here. Kinahan (1862) originally re-

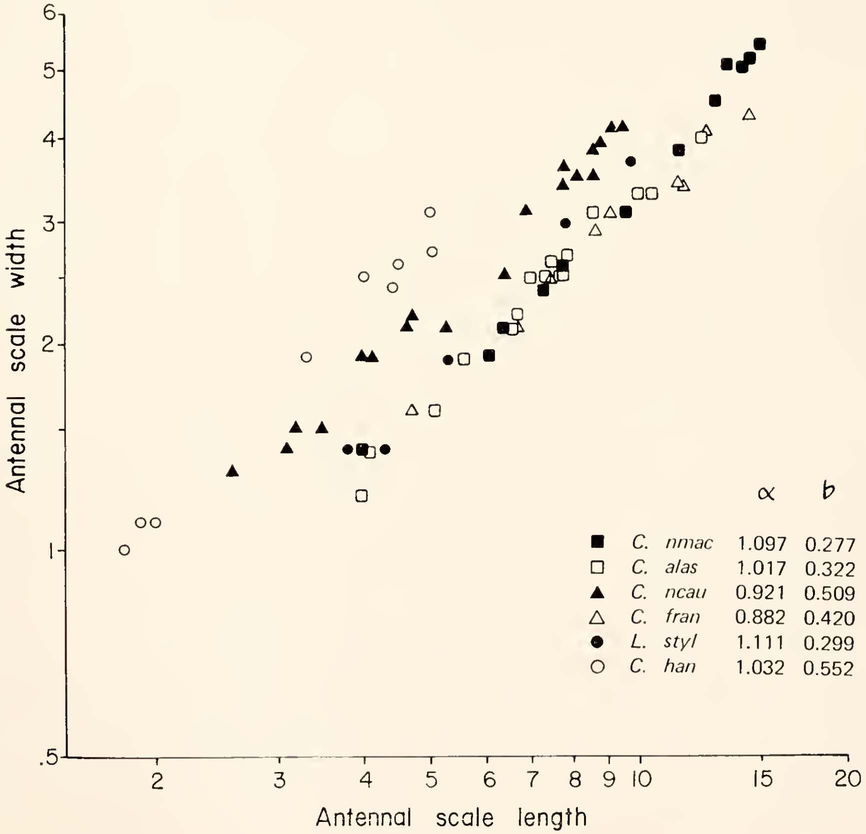

FIGURE 8. Relative growth of log antennal scale width compared with log antennal scale length for six crangonid species. See Figure 3 for key to species, abbreviations and sample sizes.

served this genus for species with either one or two dorsal keels and a sulcate dorsal surface of the telson. The name remains available for *C. allmanni* as the rest of Kinahan's listed species are included in the subgenus *Crangon* as defined above.

Neocrangon Zarenkov, 1965

Type species: *Crangon communis* Rathbun, 1899.

Generic diagnosis: Two medial gastric spines on carapace. Sixth abdominal segment with two prominent dorsal carinae. Rostrum long (extending beyond eyes), or short recurved (ascending), sometimes ornamented with a compressed ventral process.

Remarks: *Neocrangon resima* (Rathbun, 1902), *N. abyssorum* (Rathbun, 1902), *N. joloensis* (De Man, 1929), and *N. zacae* (Chace, 1937) are also included here.

Acceptance of Zarenkov's *Neocrangon* (originally proposed as a subgenus of *Crangon*), which he based upon *Crangon communis*, requires that the genus be recognized as having two gastric spines (possessed by *communis*) rather than one as Zarenkov indicated. All other species placed in *Neocrangon* by Zarenkov (and accepted without comment by Squires and Figueira, 1974), other than those noted above, must be reassigned.

DISCUSSION

The functional significance of body proportion and of body shape of shrimp is poorly understood. Shrimp anatomy may be considered through analogy with fish functional anatomy, which is relatively well-known (Marshall, 1966; Alexander, 1967; Gosline, 1971). A fast swimming fish is elongate, has a streamlined head, long, narrow caudal peduncle and lunate tail. The power stroke is delivered through the alternate contraction of the body musculature which whips the caudal fin in alternating lateral directions. Slow swimming fishes have squat bodies with short, wide caudal peduncles and a fan-shaped tail.

Shrimp swim in two different ways. Slow swimming, head-first, is achieved through the sculling action of the natatory pleopods and will not be considered further here. Rapid swimming is backwards, functioning as an escape response. The motive force here is delivered through the dorsoventral flexure of the abdominal musculature. The expanded fanshaped uropods and telson, and the sixth (last) abdominal segment, are analogous to the caudal fin and caudal peduncle, respectively, of fishes.

When carapace length is compared simultaneously with total length and with the length of the sixth abdominal segment (Figs. 3, 4) *Crangon handi* and, to a lesser extent, *Lissocrangon stylirostris* are more robust than the remaining four species. These two species also have the shortest sixth abdominal segments. Thus *C. handi* and perhaps *L. stylirostris* would seem less capable of rapid, sustained swimming than the other four species. These two squat shrimp appear to be built like sculpins (Cottidae) designed for quick short bursts of movement. Such adaptations may be related to the specialized habitats of these two species. *Crangon handi* appears to be largely restricted to intertidal and shallow shelf coarse-grained sediments. *Lissocrangon stylirostris* is also a habitat specialist, occurring as a common inshore species along high energy sandy beaches; infrequently it is captured over mud or coarse sand (Schmitt, 1921; personal observations). For both species, a squat body form with a resultant short-distance escape response and rapid reburial may be efficient to elude predators, presumably fishes.

The remaining four species with relatively attenuate body shapes are habitat generalists, found over sand and mud with a wide depth range (Schmitt, 1921; personal observations). All frequent bays, but *C. franciscorum* is rarely taken offshore, where *C. alaskensis, C. nigricauda* and *C. nigromaculata* are commonly taken. *Crangon nigromaculata* is the most elongate of these four species and also reaches the largest body size (based on examination of several thousand specimens at CAS and BMLSC).

When chela length and width are considered with reference to carapace length *C. franciscorum* and *L. stylirostris* represent the morphological extremes (Figs. 5, 6). The remaining four species are similar and intermediate with regard to chela size and shape. *Crangon franciscorum* has the longest and narrowest chela. The subchelate appendage is also the most extremely reflected; when closed, the dactyl is directed posteriorly considerably more than 45°. *L. stylirostris* has the shortest and widest chela. The anterior margin of this appendage is also the most transverse. Although the function of these appendages in *Crangon* is unknown, they may be used in feeding. If so, *C. franciscorum* and *L. stylirostris* are predicted to be feeding specialists when compared to the other four species.

The relationships between carapace length and antennal scale length and width show considerable variability between species (Figs. 7, 8). *Crangon handi* differs considerably from the other five species in having short, wide antennal scales. *Crangon nigricauda* has the next shortest and widest antennal scale. However, the slopes of the *C. handi* relationships show that the relative length of the antennal scale decreases with increasing size, while the relative width increases. The slope of the carapace length-antennal scale length relationship ($\alpha = 0.887$) falls short of the 0.9 to 1.1 range defined above as isometric and thus is characterized as negatively allometric. *Crangon nigricauda* on the other hand shows a tendency to have relatively narrow antennal scales with increasing size ($\alpha = 0.921$) and a carapace length-antennal scale length relationship that approaches perfect isometry ($\alpha = 0.965$). Of the remaining species, *C. franciscorum* has short but narrow antennal scales; antennal scale width shows a negative allometric relationship to antennal scale length ($\alpha = 0.882$). Thus the antennal scale of *C. franciscorum* is increasingly slender at larger sizes, while the scale of *C. handi* becomes increasingly wider.

Crangon alaskensis and *L. stylirostris* have relatively long and narrow antennal scales. While both relationships for *C. alaskensis* are isometric (Figs. 7, 8), the antennal scales of *L. stylirostris* are positively allometric ($\alpha = 1.111$) for the latter relationship. *Crangon nigromaculata* is similar to the two preceding species; however, visual inspection of both relationships suggests that they are curvilinear. Large specimens of *C. nigromaculata* (> 10.0 mm carapace length) have shorter and wider antennal scales than would be predicted by extrapolation from smaller specimens.

The variability in shape of the antennal scales within and between species suggests that these are important adaptive structures. Unfortunately the function of the antennal scales is so poorly understood that further discussion is not possible.

Crangon handi is remarkably cryptic when observed over the coarse grained sandy substrates that characterize the two localities where it has been observed in the field (Fig. 2a, d). This adaptation is shared by another epibenthic macrocrustacean common over the coarse-grained substrate of these localities, *Tecticeps convexus*, a sphaeromatid isopod (Fig. 2b, e). *Lissocrangon stylirostris* (Fig. 2c) and *C. nigricauda* (Fig. 2f) are normally taken over sandy or muddy substrates. These latter species are unable to match the degree of crypsis achieved by the coarse-grained specialists *C. handi* and *Tecticeps*. The black and white chromatophores of the fine-grained species are evenly distributed over their dorsal surface, creating a salt-and-pepper effect (Fig. 2c, f, i). *Crangon handi* and *Tecticeps* have a wider range of colored spots with an uneven distribution over their dorsal surface, suggesting the irregular blotchy pattern of the coarse sandy beach (Fig. 2a, b, d, e). Figure 2a and b show that this color pattern is particularly effective in disrupting the body outline, an important predator avoidance color adaptation (Wickler, 1968).

Specimens of *C. handi* and *Tecticeps* captured at Horseshoe Cove show a degree of crypsis on the light-colored coarse-grained granitic sand of the cove that is comparable to the Shell Beach specimens (Fig. 2a, b). Efforts to obtain these species from Horseshoe Cove for photographic purposes in the summer of 1975 were unsuccessful. Shell Beach specimens were placed in aquaria containing Horseshoe Cove sand 14 days prior to photography. Figure 2g and h show that adaptation to the light-colored substrate with similar grain size was only partially successful. *Lissocrangon stylirostris* collected from Horseshoe Cove does not show any ability to mimic the coarse-grain size (Fig. 2i) retaining its salt-and-pepper coloration.

Interestingly, *C. nigromaculata* is the only species considered here that presents a single distinctive lateral color spot on the posterior portion of the sixth abdominal segment. This prominent circular spot has a blue center surrounded by a black ring and then a peripheral yellow ring. On morphological evidence *C. nigromaculata* may spend more time in sustained swimming or in exposed crawling over sandy and muddy substrates. If so, it may be prone to predation from pelagic fishes (which are predators of *Crangon* spp., Skogsberg, 1939; Boothe, 1967; McKechnie and Fenner, 1971; Prince and Gotshall, 1976) in addition to bottom feeders such as flounder (also important predators of *Crangon* spp., A.M.K., personal observations; Gordon, 1974; Kosaka, 1970; see also references in Zarenkov, 1965). The lateral caudal eyespot may function as a predator shock signal or a target decoy as suggested for similar color markings on fishes (Wickler, 1968). The adaptive function of this prominent color marking in the caudal region of a posteriorly directed swimmer (while in the escape response) must be considered speculative until subjected to behavioral analysis.

Based on field and laboratory observations, it can be suggested that *Crangon handi* has adopted two distinctive adaptations compared to those of its four congeneric species. To escape detection, at least in its coarse-grained environment of the central California coast, it utilizes a squat body form to effect short distance movement in rapid bursts, followed by rapid reburial combined with crypsis, in the form of camouflaging coloration to effect body outline disruption (Fig. 2a, d). The isopod *Tecticeps convexus*, which co-occurs with *Crangon handi* in Horseshoe

Cove and at Shell Beach, appears to have adopted an even broader suite of presumed predator avoidance mechanisms. Besides a squat body form for quick bursts of movement and rapid reburial and crypsis (like *C. handi,* also remarkable camouflaging coloration on a coarse-grained background), *Tecticeps* is also capable of: modified conglobation, the isopod curling response, modified in *Tecticeps* for folding only in half, rather than into a complete ball, as in some other sphaeromatids; a probable allelochemic response, dramatic release of a cucumber (*Cucumis*)-like scent upon being handled (an allomone and/or kairomone, or depressant—see Whittaker and Feeny, 1971); spine display, the lateral protrusion of the exopod of the uropod to form protective spines when the animal is folded (Richardson, 1899); and escape maneuvering, sudden and rapid turning of direction with circular movements before reburial. Sculpins (cottids) are also common epibenthic organisms in these habitats and exhibit comparable modifications: they are squat with short, wide caudal peduncles and a fan-shaped tail; and they swim in short, quick bursts and often exhibit strong cryptic coloration. This broad suite of mechanisms suggests that predation pressure upon epibenthic fish and macrocrustaceans over exposed coarse-grained flat bottoms may be relatively high.

We thank Cadet Hand, Director of the Bodega Marine Laboratory, for the use of the laboratory's facilities and for his keen interest in the local fauna. Robert Sikora obtained the first specimen from Horseshoe Cove using SCUBA, recognized its individuality and brought the specimen to AMK. Janet Stensrud collected the first specimen from Shell Beach and brought it to our attention. John Born and Jon Standing commented on the manuscript; James Rutherford, Robert Warner, Bonnie Dalzell, Deborah Fishlyn, and John Chapman provided insightful discussions; John Cornell helped with the statistics; and Thomas Ronan, James Rutherford, and Malcolm Erskian assisted with field work. John Chapman identified the amphipods from the stomach contents. The University of California, Berkeley, Scientific Photography Laboratory took the photographs. Deborah Fishlyn aided in preliminary drawings of the shrimp; Emily Reid drew Figure 1; Sheelagh Fuzessery drew Figures 3–8. F. G. Hochberg (SBMNH), Richard Brusca, John Garth and Mary Wicksten (AHF) and Dustin Chivers (CAS) graciously provided access to the collections under their care.

SUMMARY

1. *Crangon handi,* new species, is described and compared with five other California crangonid shrimps; it occurs from the littoral zone to 55 m from Sonoma County to Bahia Colnett, northern Baja California. *Crangon handi* is a specialist for shallow-water coarse sand substrates and here exhibits remarkable crypsis. *Crangon handi* eats small gammarid amphipods and has in turn been found in the stomach of the thornback skate.

2. *Lissocrangon,* new genus, is proposed to accomodate *C. stylirostris,* a species lacking gastric spination; *Crangon* is restricted to shrimp with one gastric spine; *Steiracrangon* Kinahan, is revived for shrimp with bicarinate sixth abdominal segments; and *Neocrangon* Zarenkov is revised and amended to include shrimp with two gastric spines and bicarinate sixth abdominal segments.

3. Morphological analyses and relative growth are presented and discussed for all six crangonids considered. *Crangon handi* and *L. stylirostris* are squat shrimps in restricted habitats probably adapted for short quick bursts of movement followed by rapid reburial; the remaining four species are relatively elongate and habitat generalists.

4. *Crangon* species normally occurring on finer mud and sand bottoms are unable to achieve the degree of crypsis of the coarse-grained species, *Crangon handi*. *Crangon handi* from dark coarse-grained sediments is only partially able to adapt to light coarse-grained sediments.

5. *Crangon nigromaculata* is the only species with a lateral color spot, and it is suggested that this may serve as a predator shock signal or target decoy analogous to similar markings in some fish.

6. Fish, such as cottids, and macrocrustaceans, including *C. handi* and the sphaeromatid isopod, *Tecticeps convexus*, sympatric with it on coarse sand bottoms, exhibit a wide range of mechanisms apparently to escape detection and avoid predation. *Tecticeps* alone has at least six such mechanisms. This suggests that predation pressure in such habitats may be relatively high.

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GENETIC VARIATION WITHIN AND BETWEEN GEOGRAPHICALLY SEPARATED POPULATIONS OF THE SEA URCHIN, *ARBACIA PUNCTULATA*

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The sea urchin, *Arbacia punctulata*, is one of the most widely studied organisms in developmental and cellular biology. Knowledge of its development from the fertilized egg through the pluteus stage is considerable and continually increasing. What is understood of the genetics and population biology of this species is, however, limited and dates back primarily to the early works of Jackson (1912, 1927), Mortensen (1935), and Harvey (1956). The species is a benthic marine invertebrate, dioecious and with an enormous reproductive potential. Harvey (1956) reports that a single female contains eight million eggs during a reproductive season. Fertilization takes place in the water column, where the fertilized egg undergoes a series of cleavages, forming a blastula. Gastrulation follows and within 24–48 hours a free-swimming pluteus larva is formed which feeds on the plankton. Metamorphosis into the adult form may occur after 3–8 weeks (Cameron and Hinegardner, 1974; Harvey, 1956; Marcus, 1976). The duration of the larval phase is dependent on various environmental factors such as food and temperature (Hinegardner, 1969, 1975).

As is characteristic of many marine organisms, *A. punctulata* has a latitudinally broad range. Its recorded geographic distribution is from Woods Hole, Massachusetts, U. S. A. to Venezuela, South America, including Trinidad, Tobago, Curacao, and the Yucatan (see Harvey, 1956). The distribution of the species is not continuous within this range, however, which may be due to several factors such as low salinity and unsuitable substrates (see Marcus, 1976; Hedgepeth, 1953). Throughout this extensive range, the genus *Arbacia* is represented by a single polytypic species, *punctulata* (Mortensen, 1935). The possible existence of physiological races, ecotypes, or subspecies has not previously been examined for this species. The question that arises is whether or not any exchange between the various populations is occurring, and, if so, what effect it has on maintaining genetic continuity within the species. Exchange could be achieved by dispersal of the planktonic larval stage and, within a much more limited area, by adult migration. Morphological variation between geographically distant populations has been described (Jackson, 1912, 1927; Clark, 1923). These morphological differences may reflect basic genetic differences between the populations. On the other hand, because environmental conditions are not necessarily similar, the variation may be environmentally induced.

The present study was initiated to characterize the genetic composition of geographically separated populations of *A. punctulata*, as shown by protein variation, and to relate any observed patterns of genetic variation to environmental factors and possible routes of larval transport. It is necessary to discriminate between genetically and environmentally determined phenotypic traits before an understanding of evolutionary divergence within a species is possible.

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MATERIALS AND METHODS

Genetic variation, inferred from protein variation was determined in four geographically separated populations of *Arbacia punctulata* from: Woods Hole, Massachusetts (collected in January, 1976, by the Marine Biological Laboratory Supply Department); Wall, New Jersey (collected in December, 1975, by Connecticut Valley Supply); Willis Wharf, Virginia (collected in December, 1975, by the H. M. Terry Oyster Company); and Beaufort, North Carolina (collected in November, 1975, by N. Marcus). Upon arrival at Yale University, New Haven, Connecticut, the urchins were transferred into recirculating salt water aquaria within a constant temperature chamber at 19° C and allowed to acclimate for a minimum period of two weeks prior to electrophoresis.

At the time this study was conducted (October, 1975 to August, 1976), *A. punctulata* was not available from the Gulf of Mexico despite numerous attempts to secure them from much of the Gulf coast between Tampa, Florida and Port Aransas, Texas. The absence of the animal was attributed to a series of hurricanes occurring in the region during September and October, 1975. Repopulation of the area has been observed since November, 1976, and a small sample of urchins from the northern Gulf coast near Panama City, Florida (collected in December, 1976, by the Gulf Specimen Supply Company) has been studied. Protein variation was determined for PGM, HK, EST, and ACPH. The batch of urchins, however, were not healthy (dropping spines, absence of righting responses) and did not acclimate for a full two weeks.

Sample preparation

Each tissue sample was prepared from a freshly-killed sea urchin. The Aristotle's lantern (feeding apparatus) was removed from a slit made in the peristome, and the digestive tract was lifted out. The gut tissue was washed in extraction buffer (0.1 M Tris, pH 7.0—1.0 mM EDTA—25.0 mM 2-Mercaptoethanol—50.0 μM NADP$^+$) (Levin, Howland, and Steiner, 1972). Interference from food pellets was reduced by starving the animals during the acclimation period prior to electrophoresis. The tissue was blotted, and a portion (65.0–85.0 mg) was transferred to a polystyrene microcentrifuge tube (capacity 0.4 ml) containing 0.1 ml extraction buffer. The tissue was homogenized with a motor driven Plexiglass rod, and then centrifuged at 39,100 × g for 15 minutes. A portion (2.5 μl) of the clear supernatant was added to each gel slot (see below). The sequence of preparative steps outlined above were carried out over ice or under refrigeration. At the time of sample preparation each urchin was sexed. The size distribution of urchins examined was similar for all populations.

Gel preparation

Separation of proteins in the tissue homogenates was achieved with a horizontal microacrylamide gel apparatus (Ogita, 1975). Gels (1.0 mm thick) were made one day prior to electrophoresis and refrigerated until needed. The gels were prepared from the following stock solutions: 47.5 g acrylamide, 2.5 g bisacrylamide in 250.0 ml deionized water; gel buffer; 1.0 ml of TEMED in 100.0 ml deionized water; 240.0 mg ammonium persulfate in 100.0 ml deionized water; and deionized water. Gels containing 5.0% or 6.5% (in parenthesis) acrylamide were prepared by mixing the stocks in the following proportions: 5.0 (6.5) : 5.0: 5.0: 2.5: 2.5 (1.0).

Gel and electrode buffers

Five buffer system combinations were used: 1) gel buffer—0.11 M Tris, 0.062 M boric acid, 0.008 M NaCl pH 8.65 (Ogita, Yale University, personal communication), electrode buffer—0.3 M borate pH 8.0 (Shaw and Prasad, 1970); 2) gel buffer—0.18 M Tris, 0.1 M boric acid, 0.013 M NaCl pH 9.1, electrode buffer—0.3 M borate pH 8.0 (Shaw and Prasad, 1970); 3) gel buffer—0.32 M Tris glycine pH 8.8, electrode buffer—0.1 M glycine pH 8.6; 4) gel buffer—0.2 M Tris glycine pH 8.8, electrode buffer—0.1 M glycine pH 8.6; and 5) gel buffer—0.25 M Tris HCl pH 9.0, electrode buffer—0.5 M Tris HCl pH 9.0 (modified Shaw and Prasad, 1970).

Electrophoresis and enzyme detection

A single gel containing eight samples was inverted over buffer trays in a refrigerator at 2° C and electrophoresed at a constant voltage. Bromphenol blue and bovine serum albumin were used as the tracking standard.

At the conclusion of electrophoresis, the gel was removed and tested for enzyme activity by substrate specific staining at 37° C. Subsequently the gel was washed in water, soaked for one hour in glycerine (5.0%), and preserved permanently between two sheets of cellophane (Ogita, 1975).

Each tissue sample was tested for twelve enzyme systems. The electrophoretic conditions for each enzyme system (gel percentage, buffer system, magnitude of electrophoresis, and migration distance of the standard) are indicated below with the appropriate assay. The following assays were modified from Ayala, Powell, Tracey, Mourao, and Perez-Salas (1972): alkaline phosphatase (ALK), 6.5%, buffer system one, 70 volts, 34.0 mm, stain—25.0 mg α-napthyl phosphate, 25.0 mg Fast Blue BB, 20.0 mg $MnCl_2$, 20.0 mg $MgCl_2$, 500 mg NaCl, in 25.0 ml 0.05 M Tris pH 8.5; acid phosphatase (ACPH), 6.5%, buffer system three, 40 volts, 30.0 mm, stain—25.0 mg α-napthyl acid phosphate, 25.0 mg Fast Blue BB, in 25.0 ml 0.125 M acetate buffer pH 5.0; malic enzyme (ME), 6.5%, buffer system three, 40 volts, 33.0 mm, stain—5.0 mg $NADP^+$, 5.0 mg NBT, 1.5 mg PMS, 30.0 mg L-malic acid, 10.0 mg $MgCl_2$, in 25.0 ml of 0.05 M Tris buffer pH 9.2; malate dehydrogenase (MDH), 6.5%, buffer system three, 40 volts, 33.0 mm, stain—10.0 mg NAD, 5.0 mg NBT, 1.5 mg PMS, 30.0 mg L-malic acid, in 25.0 ml 0.05 M Tris buffer pH 9.2. The following enzyme assays were modified from Shaw and Prasad (1970): hexokinase (HK), 5.0%, buffer system five, 40 volts, 45.0 mm, stain—40.0 mg glucose, 7.0 mg $NADP^+$, 5.0 mg MTT, 20.0 units glucose-6-phosphate dehydrogenase, 1.5 mg PMS, 10.0 mg $MgCl_2$, in 25.0 ml of 0.05 M Tris buffer pH 8.0; phosphoglucomutase (PGM), 5.0%, buffer system five, 40 volts, 45.0 mm, stain—200.0 mg glucose-1-phosphate, 5.0 mg $NADP^+$, 5.0 mg MTT, 20.0 units glucose-6-phosphate dehydrogenase, 1.5 mg PMS, 60.0 mg $MgCl_2$, in 25.0 ml 0.05 M Tris buffer pH 8.0;

phosphoglucoisomerase (PGI), 5.0%, buffer system one, 70 volts, 33.0 mm, stain—40.0 mg fructose-6-phosphate, 2.5 mg NADP$^+$, 2.5 mg MTT, 20.0 units glucose-6-phosphate dehydrogenase, 0.5 mg PMS, 20.0 mg $MgCl_2$, in 25.0 ml 0.05 M Tris buffer pH 8.0; and peptidase (PEP), 6.5%, buffer system four, 40 volts, 33.0 mm, stain—5.0 mg 0-dianisidine, 5.0 mg L-amino acid oxidase, 5.0 mg peroxidase, 0.5 ml $MnCl_2$, 10.0 mg substrate (L-glycylleucine or L-leucylglycine), in 25.0 ml 0.1 M phosphate buffer pH 7.5. The following enzyme assay was modified from Hubby and Lewontin (1966): esterase (EST), 6.5%, buffer system one, 70 volts, 33.0 mm, stain—15.0 mg Fast Garnet GBC Salt, 0.5 ml substrate (1.0 g α-napthyl acetate in 50.0 ml deionized water and 50.0 ml acetone), in 25.0 ml 0.1 M phosphate buffer pH 6.5. The following assay was modified from DeLorenzo and Ruddle (1970): glutamate oxaloacetate transaminase (GOT), 5.0%, buffer system five, 40 volts, 45.0 mm, stain—100.0 mg L-aspartic acid, 50.0 mg α-ketoglutaric acid, 0.5 mg pyridoxal phosphate, 75.0 mg Fast Blue BB, 500.0 mg polyvinylpyrrolidine, in 25.0 ml 0.2 M Tris buffer pH 8.0. The assay for amylase (AMY) is modified from W. Anderson (University of Georgia, unpublished) and is as follows: 6.5%, buffer system two, 70 volts, 38.0 mm, stain—soak gel in starch substrate for 30 minutes (10.0 g Baker's potato starch, 4.54 g Tris, in 2000.0 ml deionized water, bring pH to 7.4 with HCl; heat solution until boiling or clear, and store at 5° C), wash in water and pour over gel 25.0 ml of stain (4.15 g potassium iodide, 6.25 g iodine, in 500.0 ml deionized water); let stand and wash in water. Gels stained for AMY could not be preserved.

RESULTS

Genetic variation in the four Atlantic coast populations of *A. punctulata* was inferred from the results of twelve enzyme assays, which gave rise to more than sixteen zones of activity. The identification of homozygotes and heterozygotes was based on staining and grouping patterns and conformance to patterns demonstrated for other sexually-reproducing animals. Presumed loci were assigned an abbreviation for the enzyme name and a numeral, if more than one zone (locus) was present. The locus nearest the anode was labelled one, the next two, and so forth in order of decreasing mobility. Presumed alleles at a locus were labelled by assigning a value of 1.00 to the tracking standard and then computing the mobility difference of each appropriate band variant, *i.e.*, an allele which migrated 5.00 mm less than the standard was labelled 0.95. Twelve zones were classified genetically (Fig. 1).

Acid phosphatase (ACPH) was identified as two zones of activity, each exhibiting multiple banding. Within the slow zone (ACPH-2), single- and double-banded phenotypes were observed. A triallelic system at a single locus was proposed to account for the variation. The three alleles designated were ACPH-$2^{0.79}$, ACPH-$2^{0.83}$, and ACPH-$2^{0.87}$. Single-banded phenotypes were interpreted as homozygotes, and double-banded phenotypes as heterozygotes. The second, faster migrating zone of activity could not be classified genetically.

Malate dehydrogenase (MDH) activity was present at several bands and was grouped into two zones. Within the slow zone (MDH-2) single- and double-banded phenotypes were observed. This variation was interpreted as a diallelic system at a single locus, with single-banded patterns corresponding to the homozygous state, and double-banded patterns to the heterozygous state. The two alleles

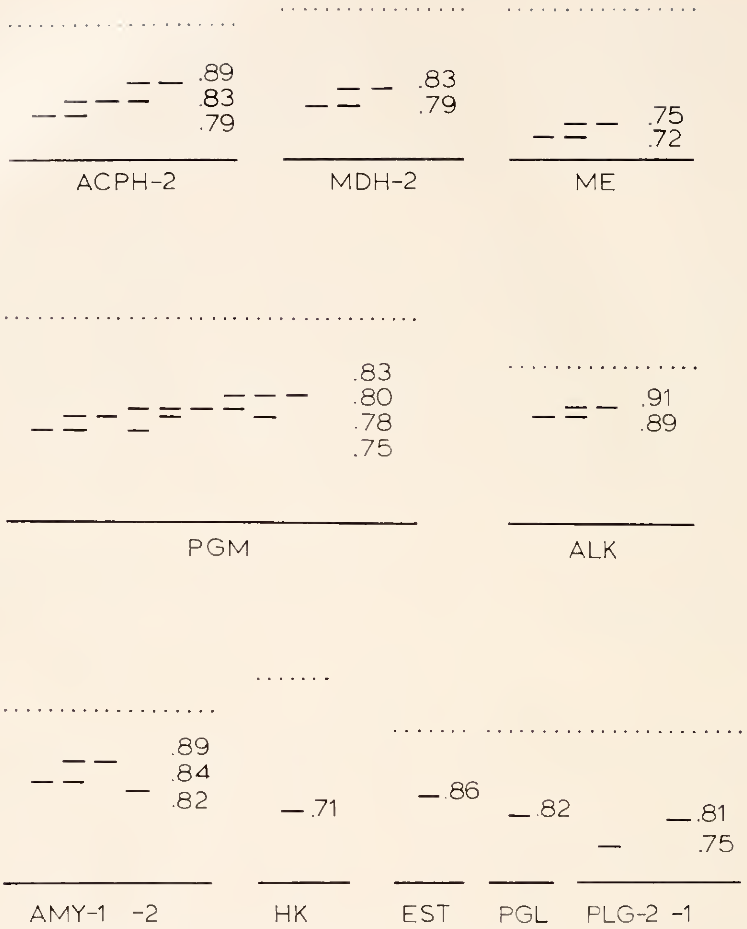

FIGURE 1. Diagram of the banding patterns for homozygotes (single-band) and heterozygotes (double-band). The origin is indicated by the solid line and migration distance of the tracking standard by the dotted line. The location of the bands and corresponding numerical value indicates the mobility relative to the standard. Designation of loci follows the text, except for PEPGL, PEPLG-1 and PEPLG-2 which are abbreviated as PGL, PLG-1, and PLG-2, respectively. The band ACPH-2$^{.89}$ is erroneously labelled and should be ACPH-2$^{.87}$.

were designated MDH-2$^{0.79}$ and MDH-2$^{0.83}$. The second faster zone could not be defined genetically.

Malic enzyme (ME) was present as a single zone of activity (ME). Single- and double-banded phenotypes were observed and were identified as homozygotes and heterozygotes respectively. Two alleles were designated, ME$^{0.72}$ and ME$^{0.75}$.

Phosphoglucomutase (PGM) was visualized as four distinct band variants within a single zone. Eight patterns were scored, consisting of either single or double bands, and these were interpreted as homozygotes and heterozygotes, respectively. The four band variants were designated allelic status as PGM$^{0.75}$, PGM$^{0.78}$, PGM$^{0.80}$, and PGM$^{0.83}$. Double-banded phenotypes expressed by individuals from

Panama City were unlike those observed in the other populations. Although the mobility of the variants corresponded to those observed for Atlantic coast specimens, the intensity of the two bands in a pair was not always alike.

Alkaline phosphatase (ALK) exhibited a complex multiple banding pattern. Only one zone of activity could be analyzed with confidence, representing a small portion of the variation detected. Within the zone of activity, single- and double-banded phenotypes were observed. These were equated with the homozygote and heterozygote classes, respectively. Two alleles were defined, $ALK^{0.89}$ and $ALK^{0.91}$.

Amylase (AMY) activity was detected as several bands which were grouped into two zones. The slow zone (AMY-2) consisted of a single heavily staining band in the majority of individuals. Fourteen urchins did not express activity in the

TABLE I

Allele frequencies and observed number of inferred genotypes for ME, MDH-2, ALK, and AMY-1. Also shown are Chi-square and probability values indicating conformance to Hardy-Weinberg equilibrium, and values of heterozygote deficiency (D_t)

ME

Population	0.72	0.75	0.72/0.72	0.72/0.75	0.75/0.75	χ^2	P	D_t
Woods Hole	0.44	0.56	1	66	9	45.20	<0.001*	0.78
Wall	0.26	0.74	0	41	38	8.81	<0.001*	0.36
Willis	0.39	0.61	4	36	16	6.19	>0.01	0.33
Beaufort	0.64	0.36	34	41	11	0.15	>0.70	0.03

MDH-2

Population	0.79	0.83	0.79/0.79	0.79/0.83	0.83/0.83	χ^2	P	D_t
Woods Hole	0.09	0.91	0	17	79	1.06	>0.30	0.06
Wall	0.26	0.74	2	36	39	3.21	>0.05	0.20
Willis	0.64	0.36	36	26	15	6.46	>0.01	−0.26
Beaufort	0.12	0.88	2	18	75	1.21	>0.20	−0.10

ALK

Population	0.89	0.91	0.89/0.89	0.89/0.91	0.91/0.91	χ^2	P	D_t
Woods Hole	1.00	0.00	95	0	0	0	>0.99	—
Wall	0.96	0.04	75	3	2	0.51	>0.30	−0.50
Willis	0.95	0.05	71	9	0	0.14	>0.70	0.18
Beaufort	0.91	0.09	65	11	1	0.10	>0.70	0.13

AMY-1

Population	0.84	0.89	0.84/0.84	0.84/0.89	0.89/0.89	χ^2	P	D_t
Woods Hole	0.58	0.42	19	75	3	33.86	<0.001*	0.60
Wall	0.57	0.43	13	64	2	31.80	<0.001*	0.64
Willis	0.60	0.40	25	37	10	0.48	>0.30	0.06
Beaufort	0.73	0.27	41	29	6	0.17	>0.50	−0.03

* Significant.

Table II

Allele frequencies and observed number of inferred genotypes for the ACPH-2 locus. Also shown are Chi-square and probability values indicating conformance to Hardy-Weinberg equilibrium, and values of heterozygote deficiency (D_t).

	ACPH-2									
Population	0.79	0.83	0.87	0.79/0.79	0.79/0.83	0.83/0.83	0.83/0.87	χ^2	P	D_t
Woods Hole	0	0.99	0.01	0	0	93	3	0	>0.99	—
Wall	0	1.00	0.00	0	0	80	0	0	>0.99	—
Willis	0.62	0.38	0.00	25	48	6	0	6.70	<0.01*	0.30
Beaufort	0	1.00	0.00	0	1	87	0	1	>0.99	—
Panama City	0	0.87	0.13	0	0	41	15	1.33	>0.20	0.15

* Significant.

zone. The pattern was assumed to represent monomorphism at a single locus, homozygous for one allele, which was designated AMY-$2^{0.82}$. The fast zone (AMY-1) consisted of single- and double-banded phenotypes, which as above were interpreted as representing homozygosity and heterozygosity at a single locus. Two allelic variants were designated, AMY-$1^{0.84}$ and AMY-$1^{0.89}$.

Hexokinase (HK), L-glycylleucine peptidase (PEPGL), L-leucylglycine peptidase (PEPLG), and esterase (EST) were monomorphic in all individuals. HK

Table III

Allele frequencies and observed number of inferred genotypes for the PGM locus. Also shown are Chi-square and probability values indicating conformance to Hardy-Weinberg equilibrium, and values of heterozygote deficiency (D_t).

	PGM				
	Population				
	Woods Hole	Wall	Willis	Beaufort	Panama City
0.75	0	0	0.01	0.07	0.02
0.78	0.10	0.05	0.11	0.22	0.13
0.80	0.10	0.13	0.33	0.25	0.21
0.83	0.80	0.82	0.55	0.46	0.64
0.75/0.75	0	0	1	0	0
0.78/0.78	5	3	5	8	3
0.80/0.80	7	11	17	16	11
0.83/0.83	50	67	29	29	35
0.75/0.78	0	0	0	7	1
0.75/0.80	0	0	0	4	2
0.78/0.80	0	0	1	1	1
0.78/0.83	3	3	1	9	8
0.80/0.83	0	0	1	1	1
χ^2	69.00	134.10	71.37	78.84	49.12
P	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
D_t	−0.85	−0.92	−0.91	−0.56	−0.61

* Significant.

TABLE IV

Chi-square and probability values for pairwise population comparisons of gene frequencies for ME, MDH-2, ALK, AMY-1, and PGM, indicating homogeneity vs. *heterogeneity of allele frequencies.*

Locus	Populations					
	Woods Hole-Wall	Woods Hole-Willis	Woods Hole-Beaufort	Wall-Willis	Wall-Beaufort	Willis-Beaufort
ME	12.74	1.01	11.24	5.73	47.05	15.18
	<0.001	>0.300	<0.001	>0.010	<0.001	<0.001
MDH-2	19.18	116.53	1.02	44.16	11.44	100.15
	<0.001	<0.001	>0.030	<0.001	<0.001	<0.001
ALK	9.52	11.55	15.77	0.26	1.92	0.78
	<0.010	<0.001	<0.001	>0.500	>0.100	>0.300
AMY-1	0.05	0.20	8.17	0.50	8.14	7.39
	>0.800	>0.500	<0.010	>0.300	<0.010	<0.010
PGM	2.16	21.33	35.29	24.27	52.49	11.68
	>0.500	<0.001	<0.001	<0.001	<0.001	<0.010

and PEPGL consistently stained for a single band which was interpreted as homozygosity for the alleles $HK^{0.71}$ and $PEPGL^{0.82}$, respectively. PEPLG consistently appeared as two bands in each urchin. I assumed the bands to represent two different loci, each monomorphic for a single allele. They were designated $PEPLG\text{-}2^{0.75}$ and $PEPLG\text{-}1^{0.81}$. The activity for EST was quite complex and only one zone could be interpreted. This zone was monomorphic for a single band and was assumed to represent homozygosity for a single allele $EST^{0.86}$.

Glutamate oxaloacetate transaminase (GOT) and phosphoglucoisomerase (PGI) activity could not be defined genetically.

An estimate of the amount of genetic variation present within each of the four Atlantic coast populations was calculated as the average heterozygosity over all loci per individual. This measure takes into account both the number of different alleles and the number of heterozygotes. The heterozygosity values for populations from Woods Hole, Wall, Willis Wharf, and Beaufort were 0.158 ± 0.314, 0.157 ± 0.279, 0.189 ± 0.260, and 0.123 ± 0.172, respectively.

For each polymorphic locus, gene frequencies were calculated, as well as Chi-square and probability values indicative of the conformance of the observed genotype distribution with expected values under Hardy-Weinberg equilibrium (Tables I, II, III). Also indicated are the values of heterozygote deficiency (D_t) (Koehn, Milkman, and Mitton, 1976). Significant deviations from Hardy-Weinberg equilibrium exist in each population, but not at all loci.

The results of a homogeneity Chi-square analysis of interpopulation gene frequency variation indicates significant deviation for five polymorphic loci (Table IV) (results for ACPH-2 not compared). At three of the loci (ALK, AMY-1, and PGM) a clinal shift in gene frequency is evident along the north-south transect from Woods Hole to Beaufort (Figs. 2 and 3). The gradual increase of $MDH\text{-}2^{0.79}$ which is evident in a southerly direction within the Virginian province, and of $ME^{0.72}$ between Wall and Beaufort (Fig. 2) may reflect clinal variation. A clinal trend was not observed for the ACPH-2 locus.

At the remaining loci (HK, PEPLG-1, PEPLG-2, PEPGL, EST, and AMY-

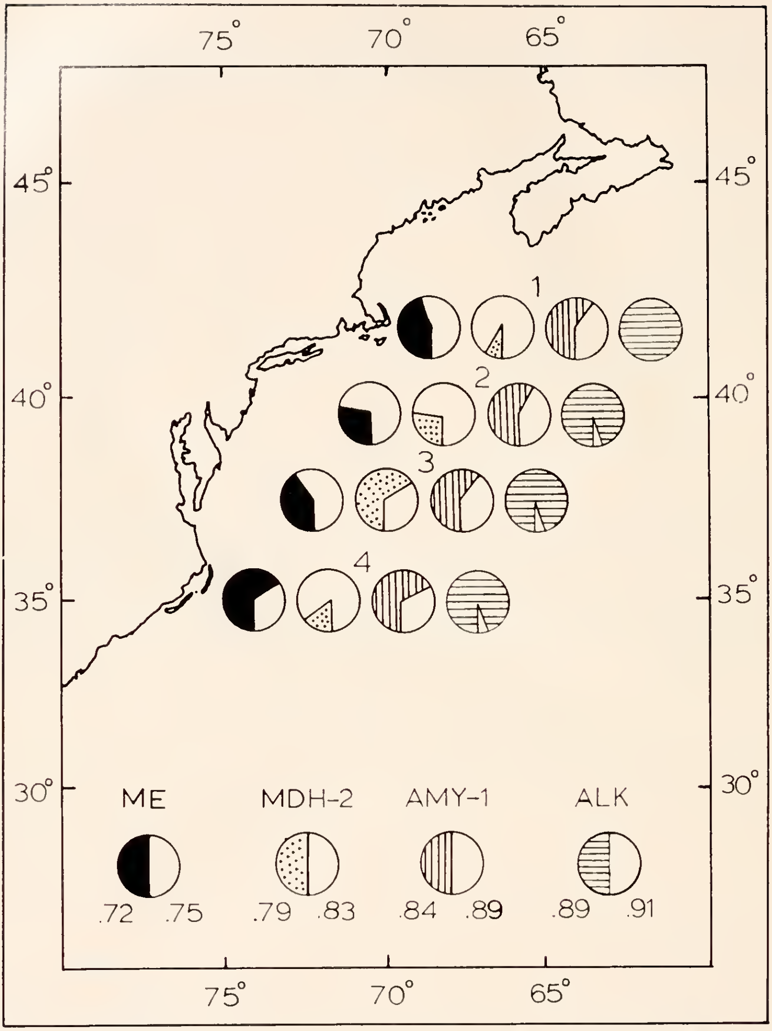

FIGURE 2. Geographic patterns of allelic variation for ME, MDH-2, AMY-1, and ALK. Designation of alleles follows the text. Numerals 1, 2, 3 and 4 correspond to Woods Hole, Wall, Willis Wharf, and Beaufort, respectively.

2), no variation within or between populations was evident. All individuals were identified as homozygotes for the same allele.

Classification of urchins according to sex was dependent upon the development of the gonads. The gonads of all specimens from Willis Wharf were reduced, and the sexes could not be distinguished. Assignment of sexual status was possible for most urchins in the other populations. This data was used to calculate gene frequencies according to sex within each Atlantic coast population for each polymorphic locus. A Chi-square test for homogeneity of allele frequencies for the sexes indicated a lack of significant variation in intersex allele frequencies.

Gene frequencies for the twelve loci (polymorphic and monomorphic) were used to determine the level of genetic identity (I′) and genetic distance (D′) (Nei, 1972) between the four Atlantic coast populations (Table V). The average genetic distance ($\bar{D}'$) for the four populations was 0.0521.

Discussion

The present study was initiated to determine the extent and pattern of genetic variation in *A. punctulata*. The data indicate significant variation in gene frequencies between the four Atlantic coast populations. Differentiation of a Gulf coast population from Atlantic populations is not striking, based upon a limited survey of four enzymes. A high level of genetic variability (0.123 ± 0.172—0.189 ± 0.260)

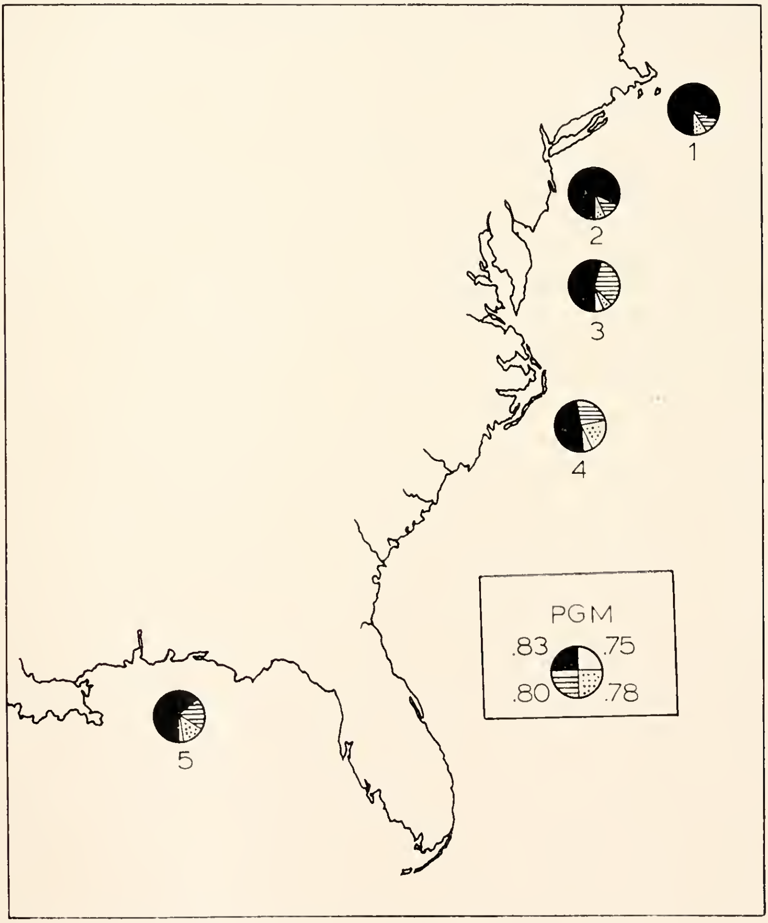

FIGURE 3. Geographic pattern of allelic variation for PGM. Designation of alleles follows the text, and of study sites Figure 2, with the addition of numeral 5 corresponding to Panama City.

TABLE V

The genetic identity (I′) above the diagonal, and the genetic distance (D′) below the diagonal are shown for each pairwise comparison of Atlantic coast populations. Also indicated is the average value of genetic distance between the four Atlantic coast populations of A. punctulata

	Woods Hole	Wall	Willis	Beaufort
Woods Hole	—	0.9929 ± 0.017	0.9171 ± 0.174	0.9812 ± 0.036
Wall	0.0071	—	0.9307 ± 0.147	0.9642 ± 0.075
Willis	0.0865	0.0718	—	0.9123 ± 0.167
Beaufort	0.0190	0.0365	0.0918	—

Average $\bar{D}' = 0.0521$.

exists within the Atlantic coast populations. The rather large standard errors for average heterozygosity indicate that the variance among loci is high, and the loci examined differ considerably in their genetic variation (see Lewontin, 1974). Heterozygosity at the ACPH-2 locus was unusually high in the population from Willis Wharf (Table II), and if this locus is excluded from the calculations of heterozygosity a value of 0.145 rather than 0.189 is obtained. All values, however, are within the range (0.024–0.202) but above the average (0.098 ± 0.022) calculated by Powell (1976) for other marine invertebrates. The higher values derived for *A. punctulata* are similar to those calculated for some plants. Selander and Kaufman (1973) report an average heterozygosity of 0.216 for four outcrossing species of Leguminosae and Compositae. A comparison of genetic variation between invertebrates and plants may be very illuminating because many sedentary marine invertebrates and plants rely on pelagic larvae or wind-borne seeds, respectively, as the primary agents of dispersal.

Several hypotheses have been proposed to account for the existence of high levels of genetic variability within and between populations (see Gooch, 1975; Levins, 1968). Powell (1971) demonstrated increased levels of genetic variability in *Drosophila* by increasing environmental heterogeneity in time and space.

The presumed adaptive nature of enzyme polymorphisms has been substantiated, although not proven, by studies which have demonstrated clinal variation in gene frequency corresponding to variation in environmental parameters (Schopf and Gooch, 1971; Murdock, Ferguson, and Seed, 1975; Johnson, 1974), selected for specific genotypes in laboratory populations exposed to different environmental regimes (Johnson, 1971; Levinton and Fundiller, 1975), and demonstrated induction of enzyme mobility variants in the same individual in response to a change in food (Oxford, 1976) and temperature (Marcus, 1977). Gene frequency clines were observed at three loci (ALK, AMY-1, and PGM) for *A. punctulata,* along the 1000 km transect between Woods Hole and Beaufort (Figs. 2 and 3). Such a trend may also typify the variation at two other loci (MDH-2 and ME). The existence of clines suggests that selection may be responsible for the maintenance of variability at the AMY-1, ALK, PGM, MDH-2, and ME loci in *A. punctulata.* The most obvious physical factor which correlates with these clinal shifts is temperature (see Bumpus, 1957). The fact that reduced clines within the full length of the transect were observed at two loci, MDH-2 and ME, indicates the interaction of several factors. It is rather doubtful that variation at different loci is the result of variation of a single environmental factor. Most likely it is the outcome of the interaction of several environmental characteristics. Migration by larval transport may promote homogeneity of gene frequencies (Thorson, 1961; Mileikovsky, 1971; Scheltema, 1971, 1972; Gooch, Smith, and Knupp, 1972), but the fact that the magnitude and direction of allelic frequency changes are not all alike, indicates that selection against unfavorable genotypes is altering the effects of migration. The effect of migration should be the same at all loci, but the outcome of selection need not be similar.

If selection is a significant factor determining the genetic structure of *A. punctulata* populations and the species as a whole, this may explain the deviations from Hardy-Weinberg equilibrium. Wallace (1968) attributes deviation from Hardy-Weinberg equilibrium to selection, migration, mutation, and sampling error. A population formed from a mixture of individuals, derived from parental stocks which differ in terms of allele frequencies, should exhibit a net deficiency of heterozygotes in accordance with the Wahlund Effect. Many benthic invertebrate populations may originate by recruitment from several populations owing to dispersal of the planktonic larval stage in the ocean currents. Examination of the present data on *A. punctulata* which does produce a planktonic larval phase, indicates that marked heterozygote deficiencies were observed for the PGM locus only. The other loci which deviated from Hardy-Weinberg equilibrium expressed small excesses of heterozygotes. These observations tend to support the viewpoint that selection is highly important in determining the genetic structure of these populations, and for the most part over-rides the 'melting pot' effect of the larval influx from different parent populations. Heterosis may be the selective mechanism involved as indicated by the excess of heterozygotes. The variability does not appear to be due to differences in the sexes as demonstrated by the homogeneity Chi-square analysis. Size is not believed to be a factor either, because the size distribution of individuals examined was similar for all populations.

Although significant gene frequency differences were evident between the four Atlantic coast populations of *A. punctulata,* the genetic distance values between populations when all loci were considered were not high (Table V). Levels of genetic distance varied from 0.0071–0.0918 and were greatest (0.0718–0.0918) when the population from Willis Wharf was considered. Genetic distance values were less (0.0071–0.0365) for the pairwise comparisons of Woods Hole, Wall, and Beaufort populations. These values are of the same magnitude as values computed for geographic populations of other species. For example Nei (1972) calculated genetic distance values of 0.002–0.03 for geographic populations of the mouse (*Mus*); Hedgecock and Ayala (1974) calculated values of 0.005–0.053 for populations of the newt (*Taricha*); and Tracey, Nelson, Hedgecock, Schleser, and Pressick (1976) report values of 0.001–0.036 for populations of the lobster (*Homarus*). Using the data of Schopf and Gooch (1971) on the marine ectoproct (*Schizoporella errata*) I calculated values of 0.014–0.053. Genetic distance values which characterize subspecies are higher. Hedgecock and Ayala (1974) report a range of 0.109–0.253 for subspecies of *Taricha*. Genetic distance values between local populations of *Drosophila* average 0.003 ± 0.006 and between subspecies, 0.228 ± 0.026 (see Ayala, Tracey, Barr, McDonald, and Perez-Salas, 1974). The values of genetic distance for the population from Willis Wharf generally are intermediate to the values for local populations and subspecies. The greater differentiation displayed by the population from Willis Wharf could be the result of geographic isolation and/or strong selection in a heterogeneous environment.

It appears that on the basis of allelic frequencies, genetic distance between Atlantic coast populations from Woods Hole, Wall, Willis Wharf, and Beaufort are within the range of values displayed by geographic populations of a single species. This continuity may be promoted by extensive larval transport. The success of larval transport as an agent of gene flow is dependent upon several factors, such as time of spawning, duration of life in the plankton, and current velocity and direction. Bumpus (1974) reports a general southwesterly drift for surface currents longshore from Nantucket Shoals to Cape Hatteras. In winter the velocity is maximal and on the order of 12 nautical miles/day. During the summer period, conditions such as prevailing southerly winds and reduced fresh water run-off may promote current reversals producing northerly drifts of approximately 5 nautical miles/day. Bottom drift at all times is mostly southerly and considerably slower than surface drift.

The length of larval life places one limit on how far an individual will be transported. Clearly larval life depends upon various factors such as temperature, food, and settlement responses. In the laboratory, a well fed pluteus of *A. punctulata* will metamorphose in as little as 22 days at 23°–25° C (Cameron and Hinegardner, 1974; Marcus, 1976). Larval life can be prolonged by cooler temperatures and by reduced feeding (Hinegardner, 1969, 1975). Hinegardner (1969) reports that a well-fed larva can live up to four months, but loses the capacity to metamorphose after two months. Gordon (1929) reports that larvae fed *Nitzschia closterium* began to metamorphose after 40 days, and Turner (1965) observed metamorphosis after 42 days. Thorson (1961) cites an unusually lengthy period of 97 days as the time to metamorphosis, but does not give a reference. If an average larval life of sixty days and a current velocity of 5 nautical miles per day is assumed, then a pluteus may be transported longshore during the summer a maximum distance of 558 km prior to metamorphosis. This distance represents more than half the transect between Cape Hatteras and Woods Hole. It is doubtful that surface temperatures that range from 20° C at Woods Hole to 25° C off Virginia at this time of the year would prolong larval life (Walford and Wicklund, 1968). On the other hand, juvenile and metamorphosing urchins have been collected in surface and oblique plankton tows (personal observation) in early September off Cape May and the coast of Delaware, where bottom depths are about 30 meters. Metamorphosis, therefore, need not signal the termination of transport by the ocean currents. Although the origins of the larvae collected are not known, transport of such stages would increase considerably the potential dispersal distance of the species. Because of the distance involved, migration between Willis Wharf and Woods Hole is likely to be less common than between Willis Wharf and Wall. The values of genetic distance for these populations support this hypothesis. The genetic distance between Woods Hole and Willis Wharf (0.0865) is larger than the genetic distance between Wall and Willis Wharf (0.0718). Larval exchange between Woods Hole and Wall is probably more common as seen in the small genetic distance between the two populations (0.0071).

Although Cape Hatteras is considered a barrier to the continuous distribution of many marine organisms (Parr, 1933), genetic continuity may be promoted by the Gulf Stream and occasional spillovers of water from the Virginian province into the Carolinian province. It is reported that northeast winds in late summer and autumn promote the flow of water south of Cape Hatteras from the Virginian province (Gray and Cerame-Vivas, 1963). The southward movement of *A. punctulata* may be promoted by this system. The Gulf Stream may provide a major avenue for the transport of larvae northward to Woods Hole from the Carolinian sector. In the region of the Carolinas, *A. punctulata* is found both inshore and offshore on reefs characterized by their more tropical faunal elements (Cerame-Vivas and Gray, 1966). The presence of tropical species is attributed to the Gulf Stream which moves in a northerly direction from the Florida region. *A. punctulata* larvae may thus enter the Gulf Stream system and be transported northward. From April through September strong southerly winds cause an intermingling of coastal with Gulf Stream water, and organisms common to the Sargasso Sea are often found along the coast of Massachusetts having been blown inshore from the Gulf Stream by the winds (Sumner, Osburn, and Cole, 1911). These same forces may result in an influx of larvae from the Carolinian province. The Gulf Stream may thus be largely responsible for the great genetic similarity of the Woods Hole and Beaufort populations analyzed in this study. The greater distance observed when Beaufort urchins are compared with those from Willis Wharf supports the viewpoint recognizing Cape Hatteras as a major barrier, through which only limited dispersal can occur.

The application of this reasoning on larval transport between regions leads to the following conclusions concerning the genetic status of the populations in the Gulf of Mexico. Information on the geological history of the Gulf of Mexico and the Florida peninsula, suggests that genetic distance between the populations of the Atlantic coast and Gulf coast should be at least as great as the difference between the Beaufort and Willis Wharf populations. The Florida peninsula must act as a barrier to larval transport as does Cape Hatteras. If it is a complete obstacle to gene flow, then the populations from the Gulf of Mexico and the Atlantic have been diverging genetically ever since the emergence of the Florida peninsula, more than 30,000 years ago (see Hedgepeth, 1953). The southern part of the Florida peninsula and the Florida Keys may represent a transition zone of limited gene exchange. *A. punctulata* is rare in this region (Hedgepeth, 1953). Hydrographic data also support the premise of limited gene exchange. Parr (1935) reports that mixing between the rapidly flowing water of the Straits of Florida and the Gulf of Mexico is limited. He identifies the origin of the Florida Current from the Caribbean. This implies that any substantial exchange between the gene pools of the Gulf of Mexico and the Atlantic does not occur.

There are many species which occur both along the Atlantic and Gulf coasts, and protein variation of some of these organisms has been studied. The horseshoe crab (*Limulus polyphemus*) is polymorphic at nine of fifteen loci examined. At all but two of these loci, there are clear and consistent differences between the populations of the two coasts (Selander, Yang, Lewontin, and Johnson, 1970). Differences have also been noted for fish (Weinstein and Yerger, 1976; Johnson, 1974), and fiddler crabs (Selander, Johnson, and Avise, 1971). On the other hand, a lack of differentiation has been observed for the white shrimp (Marvin and Caillquet, 1976). Preliminary electrophoretic data of four loci of *A. punctulata* from the Gulf of Mexico does not reveal major differences between Atlantic and Gulf coast populations. Alleles or loci unique to the Gulf population were not observed. More evidence is required before any definitive answer is reached as to whether or not the results of these few loci are representative of the entire gene pool for the Gulf of Mexico.

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SUMMARY

Genotypic variation, inferred from isozyme differences in twelve enzyme systems, was measured by means of horizontal microacrylamide gel electrophoresis in four populations of the sea urchin, *Arbacia punctulata,* from the Atlantic coast of the United States. An additional population from the Gulf of Mexico was surveyed for four enzymes. Although gene frequencies were significantly different among Atlantic populations, the overall values of genetic distance were within the range observed for geographic populations of other species. A balance between migration and selection is proposed to account for the patterns of genetic variation. Striking differences in gene frequency were not observed when Atlantic coast populations were compared with Gulf of Mexico specimens.

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STUDIES ON THE ANNUAL REPRODUCTIVE CYCLE OF THE SEA URCHIN AND THE ACID PHOSPHATASE ACTIVITY OF RELICT OVA

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Among the echinoderms, in which the oocytes grow into mature eggs during a few months before the reproductive season, the ovaries show an annual cyclic change. Nuclear changes, cortical granule formation, vitellogenesis and RNA-synthesis in sea urchin oogenesis have been studied with the electron microscope (Millonig, Bosco and Gimbertone, 1968; Anderson, 1968; Verhey and Moyer, 1967). A radial nerve factor has been found to induce spawning in echinoids (Cochran and Engelmann, 1972), and changes in the amount of 1-methyladenine in the sea urchin ovary during oogenesis have been reported (Kanatani, 1974). In spite of the important status of the sea urchin egg, there are several fundamental points in connection with the annual morphological changes of the sea urchin ovary that have not been made completely clear.

Recently, it has been found that the follicle cells of asteroidean ovaries produce 1-MA during the process of oocyte maturation (Hirai, Chida and Kanatani, 1973); but in the Echinoidea, it is suggested that there may be a dynamic relationship between the germ cells and nongerm cells, which are variously known as follicle cells, accessory cells and nutritive phagocytes (Holland and Giese, 1965; Chatlynne, 1969; Bal, 1970). The annual changes that take place in the accessory cells are poorly understood, and the function of the accessory cells is still under discussion (Takashima and Tominaga, 1975).

This study focuses on the accessory cells of *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* during the ovarian cycle, using the staging concept of Fugi (1960): stage I (spent recovering stage); stage II (growing stage); stage III (premature stage); stage IV (mature stage); and stage V (spent stage). The acid phosphatase activity of relict (unshed) ova at stage V is also observed, and a model is proposed to account for the process by which the relict ova degenerate.

MATERIALS AND METHODS

Two species of sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus,* were collected once or twice a month from May, 1971 to 1973, along the coast near the Tateyama Marine Laboratory. Ovaries were removed and tissue samples were fixed with 1% OsO_4 in sea water for one hour at room temperature. After dehydration, the samples were embedded in Epon 812. Half-micron sections were cut on an ultramicrotome, stained with 0.5% toluidine blue in phosphate buffer (pH 7.0) for 15 min at room temperature and observed by light microscopy.

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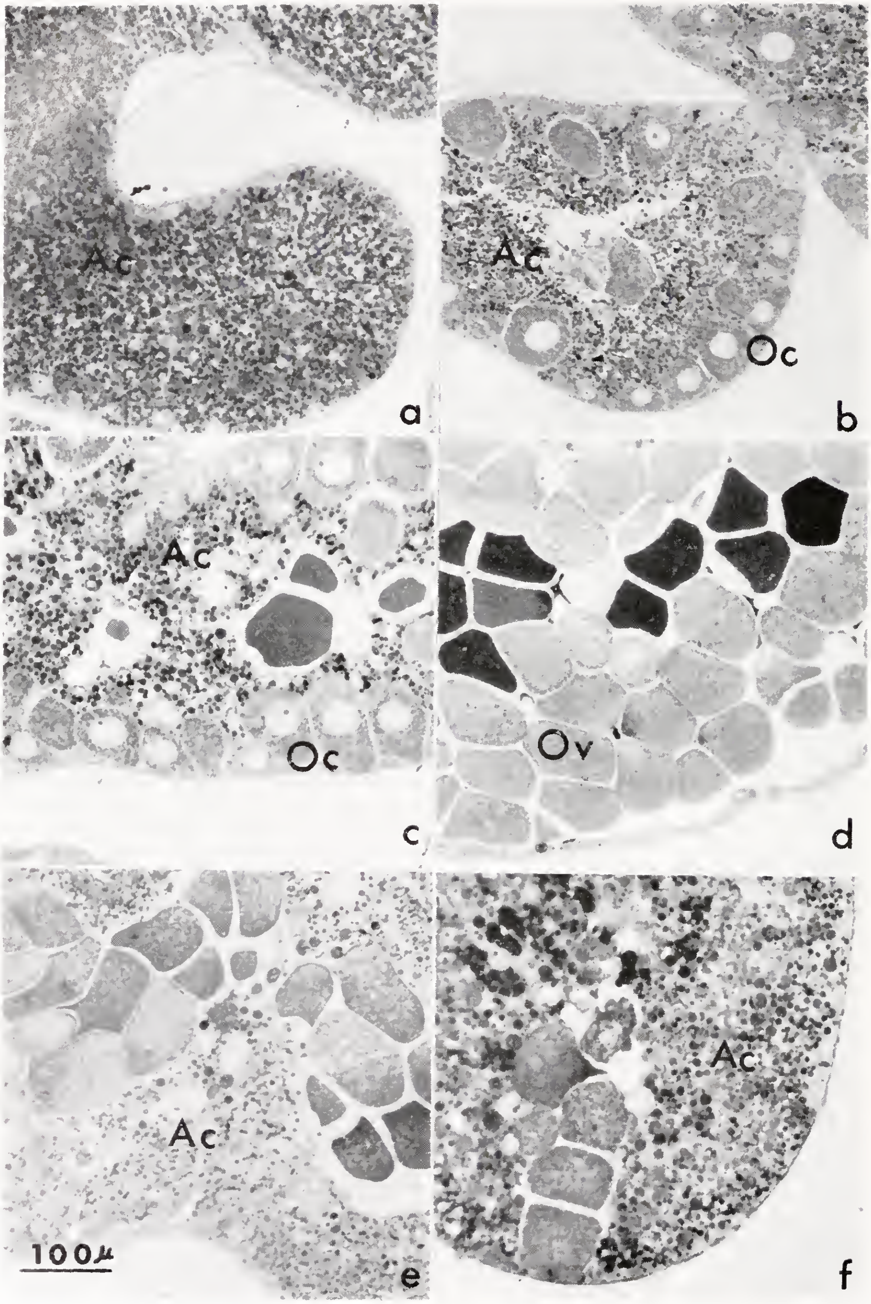

FIGURE 1. Thick sections at various stages of Epon-embedded ovaries of *Anthocidaris crassispina*, stained with toluidine blue: a, stage I, January—accessory cells including numerous globules occupy the ovariole; b, stage II, March—many growing oocytes are attached to the ovarian wall; c, stage III, June—fully grown oocytes complete meiosis; d, stage IV, August—

The gonad indices were also determined for these two species (gonad wet weight/body weight × 100). About ten samples were collected for each average index value from March, 1972 to September, 1973. Sea surface temperature data from Ninomiya and Yorogami (1974) were used.

Acid phosphatase activity was investigated, using *Anthocidaris crassispina* at stage V (spent stage). Eggs mixed with some accessory cells were spawned into sea water following introduction of 0.5 M KCl into the body cavity, and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min. Samples were incubated at 38° C for one hour in Gomori's solution [0.05 M acetate buffer (pH 5.0) 500 ml, lead nitrate 0.6 g, 3% glycerophosphate 50.0 ml], followed by washing in deionized water containing 1% yellow ammonium sulfide. Samples were embedded in Epon 812, and thick sections (0.5 μm) were made for observation with the light microscope. As control, Gomori solution without substrate (glycerophosphate) was used as the incubation medium. The same procedure was also carried out with ovaries in stage III (premature stage).

Specimens were fixed for electron microscopy with 2.5% glutaraldehyde for one hour, post-fixed for one hour with 1% OsO_4 in 0.1 M phosphate buffer, and embedded in Epon 812. Thin sections were stained with uranyl acetate and observed with an Hitachi HS-7D electron microscope.

RESULTS

Ovarian cycle

Figure 1 shows a cross-section of *Anthocidaris crassispina* ovarioles. From December to March, the main part of the stage I ovary is filled with accessory cells containing numerous globules, some of them 10 μm–15 μm in diameter. Small oocytes about 10 μm in diameter are observed along the ovarian wall (Fig. 1a). From March to May (stage II), the growing oocytes (Fig. 1b) rapidly come to contain many yolk granules of various sizes. During May and June (stage III), the full-sized oocytes undergo the reduction divisions and move toward the center of the ovariole to be stored as mature ova until they are spawned (Fig. 1c). As the reproductive season begins, the stage IV ovariole is almost completely filled with ova and a very few accessory cells can be observed along the ovarian wall (Fig. 1d). After the end of the spawning season (stage V), the amount of space occupied by accessory cells increases, and the ovary at this stage sometimes contains unshed (relict) ova which are in the process of degenerating (Fig. 1e, f).

The same morphological changes are observed in *Hemicentrotus pulcherrimus.* From May to November (stage I), the ovarioles are filled with accessory cells containing globules (Fig. 2a). The small oocytes arranged along the ovarian wall grow rapidly in November to full size (Fig. 2b, stage II). Globules are less conspicuous in the accessory cells (Fig. 2c, stage III), and the area occupied by each cell seems to be smaller than before. The gonad index is highest at this stage (Fig. 3). Sometimes "empty" accessory cells are observed around fully

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mature eggs occupy most of the lumen and flattened accessory cells line the ovarian wall; e, stage V, September—relict ova are present in the center of the ovariole, and the space occupied by accessory cells has increased; and f, stage V, December—relict ova degenerate at the center of the ovarian lumen. Ac represents the area of accessory cells; Oc, oocyte; and Ov, ovum.

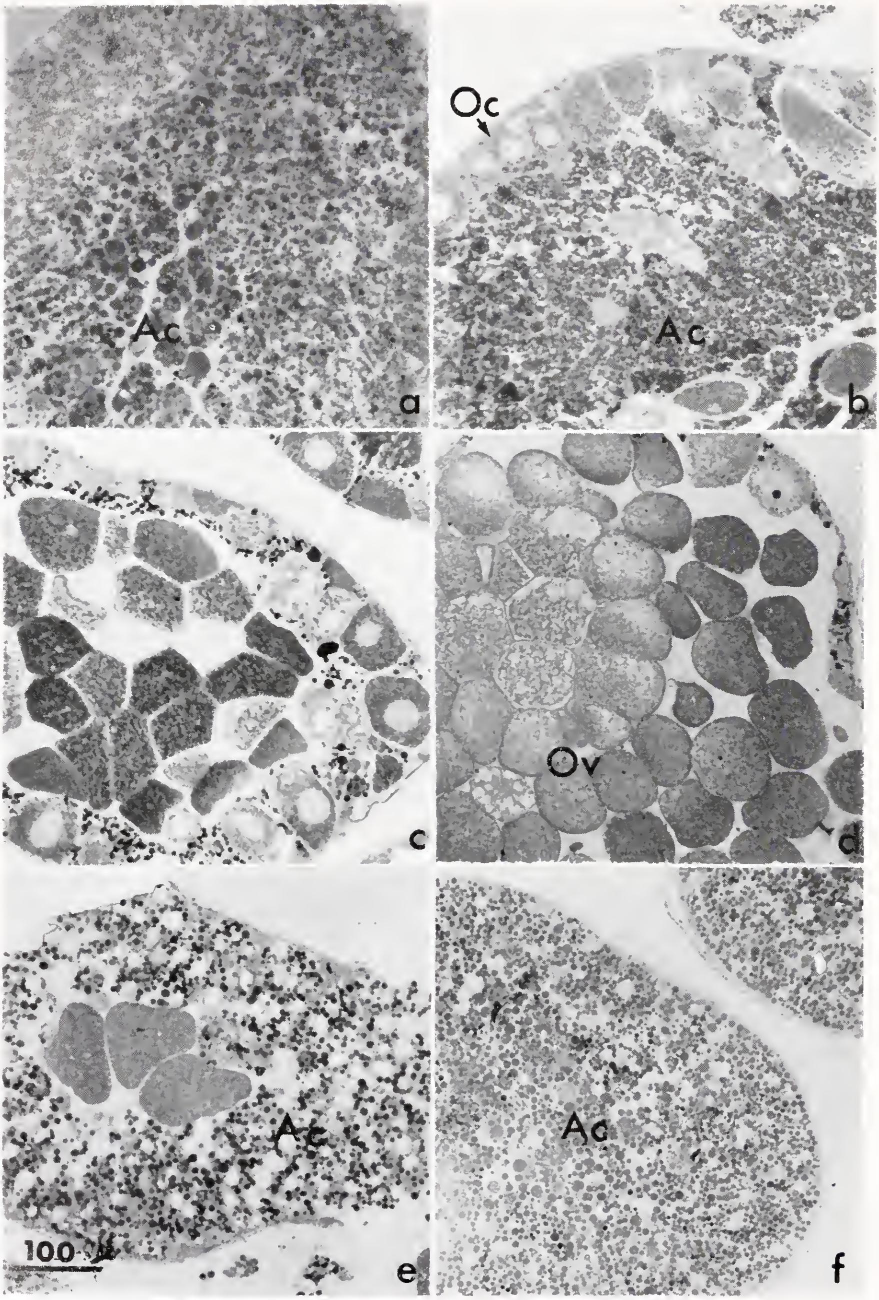

FIGURE 2. Thick sections of Epon-embedded ovariole of *Hemicentrotus pulcherrimus:* a, stage I, November—numerous accessory cells occupy the lumen and a few small oocytes are present along the wall; b, stage II, December—fully grown oocytes are seen along the wall; c, late state of stage III, January—fully grown oocytes complete meiosis, mature eggs occupy the

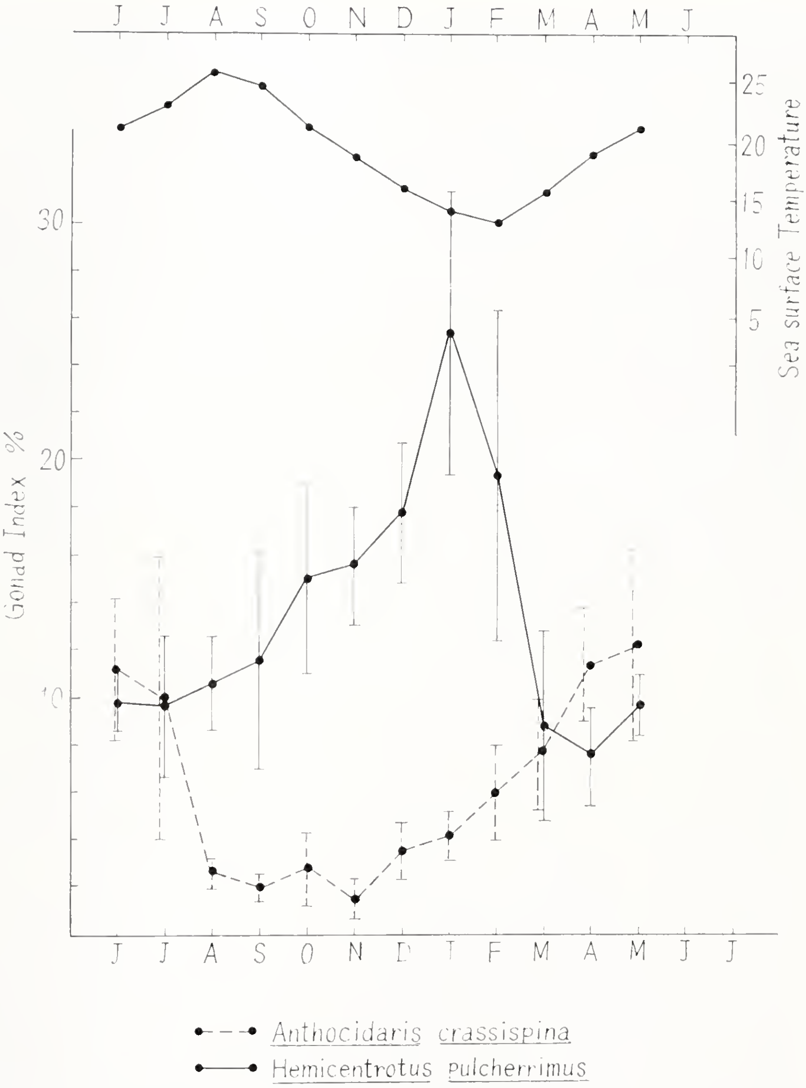

FIGURE 3. The gonad index (gonad wet weight/body temperature × 100) and sea surface temperature. Data were taken on the day of collection.

grown oocytes. Most of these oocytes complete meiosis during early December, and the mature eggs are stored in the center of each ovariole. The reproductive season (stage IV, Fig. 2d) begins in January and ends in March. Mature eggs

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lumen and a few oocytes are present along the wall; d, stage IV, February—mature eggs in the lumen, some of which are degenerating; e, stage V, April—a few relict ova are present in the center of lumen, with more accessory cells present than in the previous stage; and f, stage I, May—the same stage as Figure 2a.

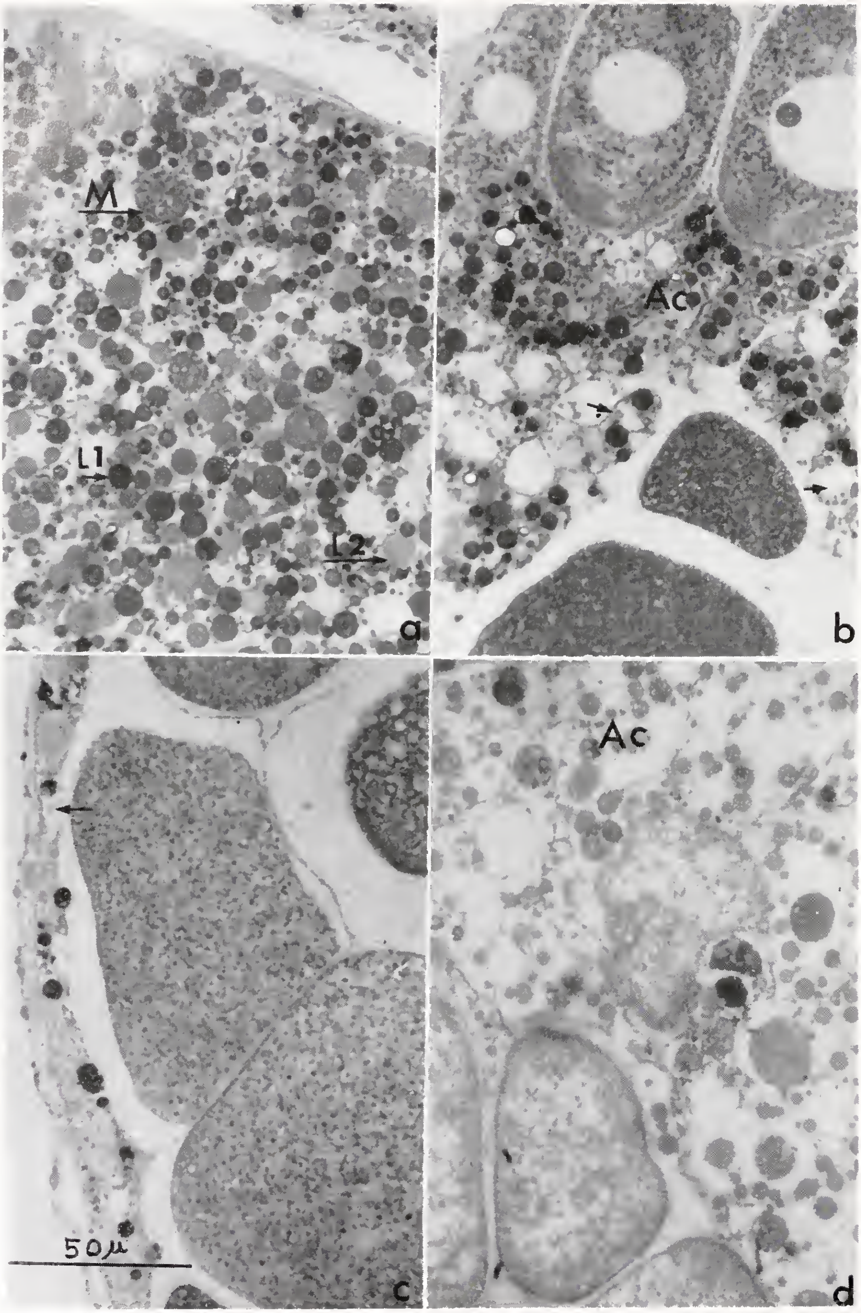

Figure 4. Various changes in the inclusions of the accessory cells in the course of the ovarian cycle in *A. crassispina:* a, stage I—accessory cell is filled with various types of globules (L1, large globule 1; L2, large globule 2; M, mosaic globule); b, stage III—empty cells

occupy the whole space within the ovary, and only a small number of "empty," flattened accessory cells can be observed near the end of the reproductive season; the relict ova remaining in the center of the ovary become vacuolated (Fig. 4d). After the reproductive season (stage V), the ovarioles are again filled with globules containing accessory cells (Fig. 2e). The layer of accessory cells gradually becomes thicker, until finally the ovarioles are completely occupied by accessory cells containing globules of various sizes. The relict ova have all degenerated by this stage (Fig. 2f).

On the basis of these observations, the annual ovarian changes of two species of sea urchins at Tateyama have been summarized following the staging of Fuji (1960). Of these species, *H. pulcherrimus* has the longer period assignable to stage I, but their ovarian cycles follow basically the same pattern, in which the gonad index (Fig. 3) shows the highest value at stage III (*H. pulcherrimus* in January, *A. crassispina* in the middle of May). The mean value of the sea surface temperature varies from 13.1° C in February to 25.9° C in August.

In *Strongylocentrotus purpuratus*, Holland and Giese (1965) reported that oogonial clusters could not be found during the winter (stages III and IV), while Chatlynne (1969) described that oogonia are very difficult to find in the recovering spent stage (stage I) but are numerous in the winter (stages III and V). According to Gonor (1973a), oogonial clusters were found at all times of the year, but from November through March (stages III and V), their numbers are low and they are easily overlooked. In the present study, the clusters of germ cells including oogonia and small oocytes were observed in *A. crassispina* at stage I, while in *H. pulcherrimus*, these cells were very difficult to observe at the same stage. The germ cells at stage I in *H. pulcherrimus* seem to be dormant.

Accessory cells

At stage I, the ovarioles are filled with accessory cells containing conspicuous globules, which can be divided into three types: large globule 1, which stains strongly with toluidine blue; large globule 2, which stains weakly with toluidine blue; and mosaic globule, which contains cortical granule-like structures. The diameters of these globules range from 3 μm–10 μm (Fig. 4a). At stage III, many "empty" accessory cells could be observed around fully grown oocytes and ova (Fig. 4b, arrow). At stage IV, the maturing stage, accessory cells are seen along the ovarian wall, and the center of the ovarioles is filled with mature eggs (Fig. 4c). After the reproductive season, the space occupied by accessory cells increases, and a few relict ova are seen at the center of the lumen (Fig. 4d). Takashima (1968) divided these globules into four types; large granule A, large granule B, large granule C and oil droplets. The large granule A corresponds to mosaic globules in the present study, large granule B to large globule 2 and large granule C to large globule 1.

Figure 5 shows a cross-section of an ovariole at stage V. At this time, mosaic globules become conspicuous in the accessory cells surrounding the degenerating

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(arrows) are seen around full-sized oocytes or ova; c, stage IV—flat "empty" accessory cells line the ovarian wall (arrow); and d, stage V—"relict" ova in which cytoplasm but not cortex appears disrupted. Accessory cells have fewer globules.

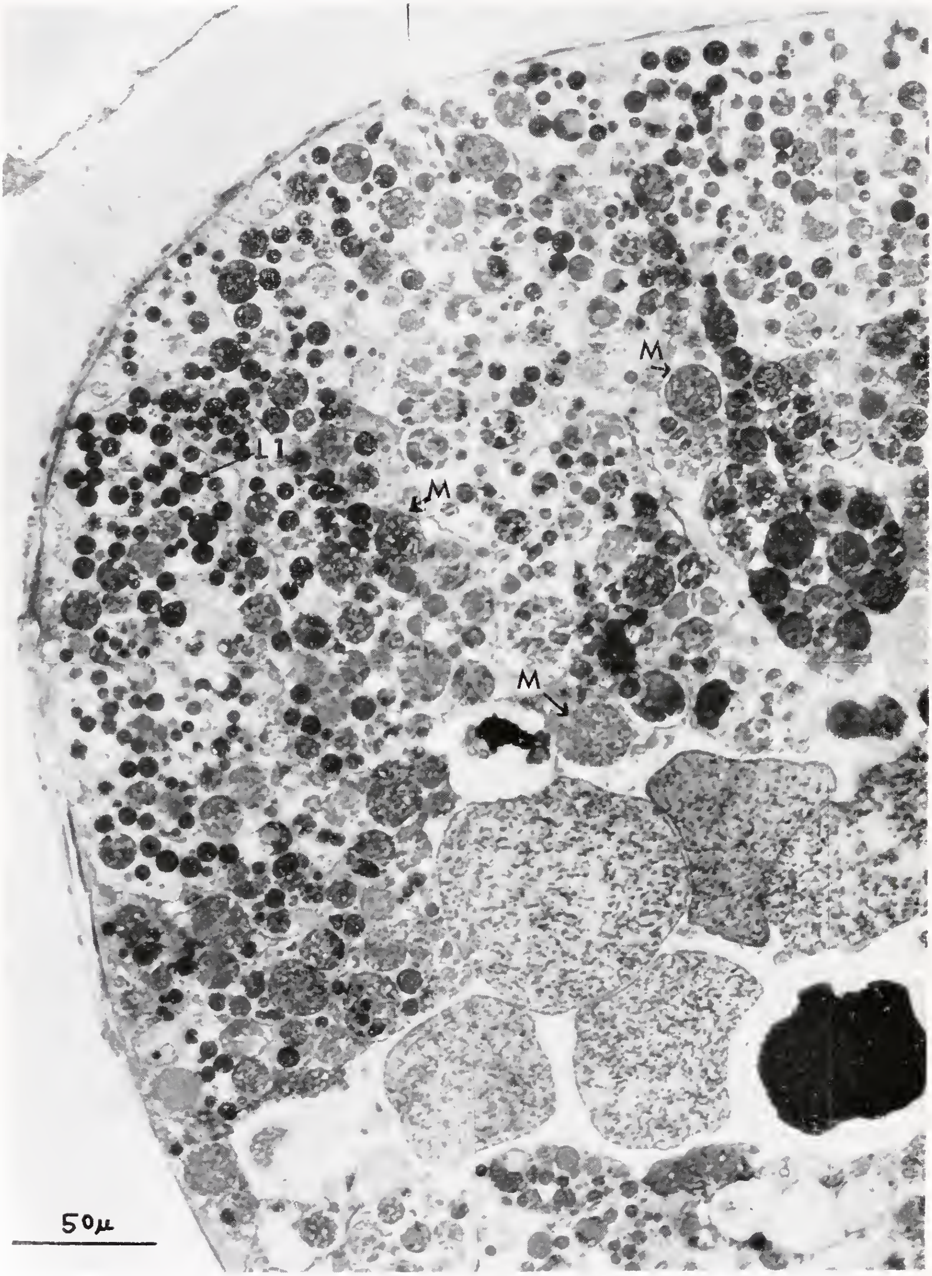

FIGURE 5. Cross-section of an ovariole of *A. crassispina* at stage V (December). Unshed (relict) ova are seen at the center of the lumen, surrounded by many accessory cells containing mosaic globules (L1, large globule 1; M, mosaic globule).

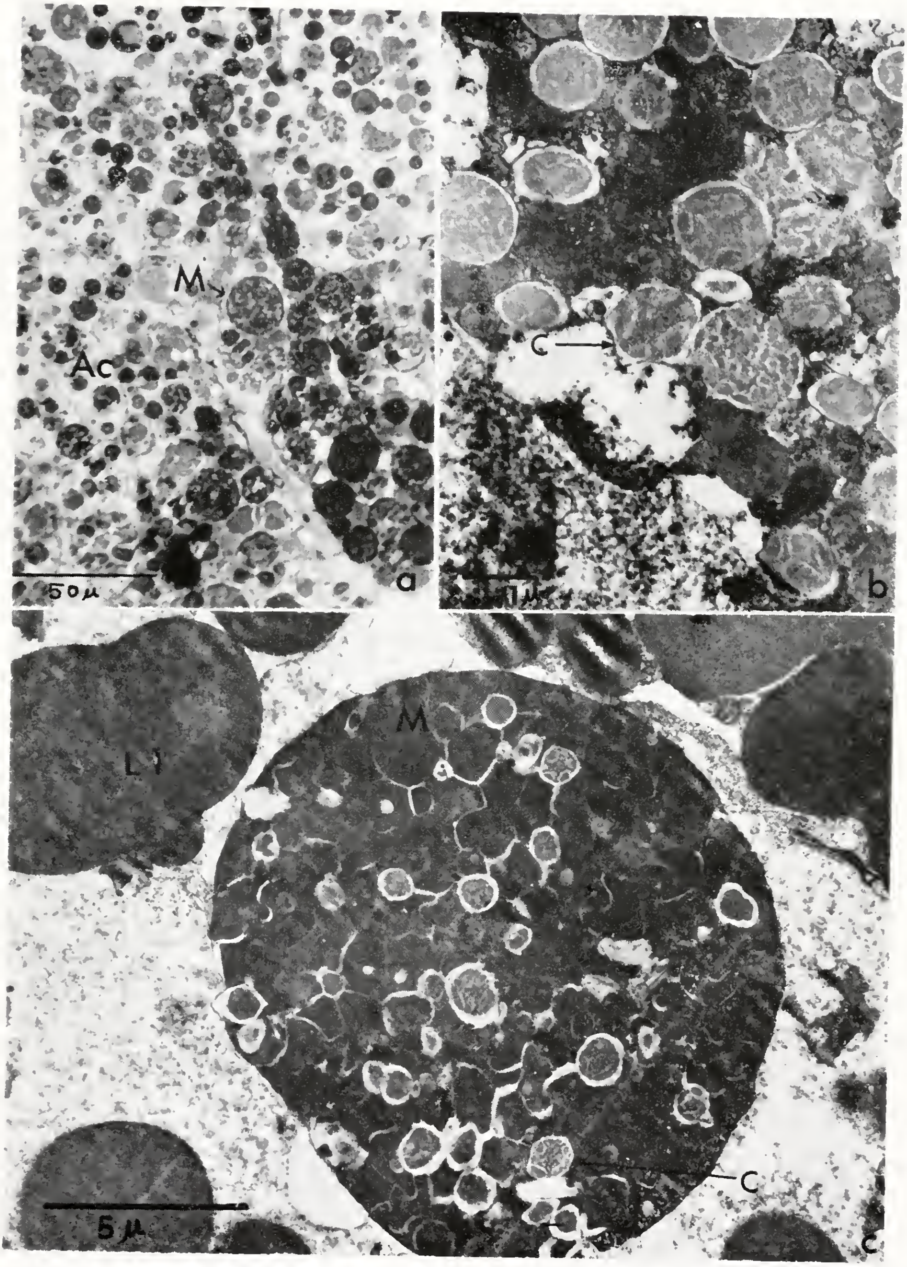

FIGURE 6. Fine structure of mosaic globule: a, light microscopic observation of mosaic globules; b, c, electron microscopic observations of mosaic globules, containing cortical granule-like structures. Ac represents accessory cell; c, cortical granule-like structure; Ll, large globule; and M, mosaic globule.

ova, the size of which is 10 μm–30 μm, and the two types of large globules, large globule 1 and large globule 2, are rarely seen compared with the state of stage I (Fig. 4a). The mosaic globules aways contain structures which are about 1 μm in diameter and have the appearance of cortical granules (Fig. 6a, b, c).

Acid phosphatase activity of relict ova

In both species, the relict ova remaining in the ovarioles at stage V undergo degenerative changes: large "empty" spaces are present in the cytoplasm, and the number of yolk granules is greatly reduced (Fig. 1f, 2d, 4d, 5); or the shapes of the cells depart markedly from the spherical (Fig. 1e, 2f, 4d). In most cases, however, the egg cortex seems to remain relatively unchanged (Fig. 4d, 5). Eggs showing these characteristics were collected and subjected to a Gomori test to detect acid phosphatase activity. Deposits of lead sulfate are observed in the ova, accessory cells (Fig. 7a) and in small oocytes (Fig. 7b). Specimens which were incubated without substrate do not show such deposits (Fig. 7c, control). In some cases (Fig. 7a, b), the phosphatase activity is localized in an irregular manner. When the same procedures were carried out with eggs collected during the prematuring stage (stage III), the acid phosphatase activity was found to be about the same as that of the control (Fig. 7d, e; control).

These results show that relict ova have strong acid phosphatase activity compared to normal ova.

DISCUSSION

Fluctuations of the food supply may explain the great variability observed in gonad size and fertility; a sufficient food supply in the gonad permits a large number of oocytes to become ova simultaneously (Boolootian, 1966). Gonor (1973a) studied the influence of environmental factors in the annual changes of the ovary. The gonad indices of *A. crassispina* and *H. pulcherrimus* collected at the coast of Tateyama (Tokyo Bay) are alike in showing a steady increase in the size of the ovary during the period of oocyte growth preceding each breeding season, but they differ conspicuously in the minimum size observed after the end of spawning. The *A. crassispina* ovary diminishes to 15% of its maximum size, as spawning ends in late summer, and remains very small through the autumn until December. The *H. pulcherrimus* ovary retains 33% of its maximum weight at the end of the spawning season in April, and immediately begins to increase slowly in late spring and summer and then rapidly through the autumn, until November (*i.e.,* the ovary is in stage I during this time when it acquires 50% of its maximum bulk). This increase of gonad weight in spring, summer and autumn means that the gonad stores nutrients and prepares for the oogenesis which will take place in December when the food supply may be insufficient. Thus, from April until November, it might be more accurate to regard the *H. pulcherrimus* ovary as a nutrient-storing, rather than a gamete-producing, organ. Gonor (1973b) observed the ovary of *S. purpuratus* and divided the annual changes into two phases: first, the period of oogonial proliferation, resulting in an increase in the number of clusters; and secondly, the period when the oogonia in clusters transform into

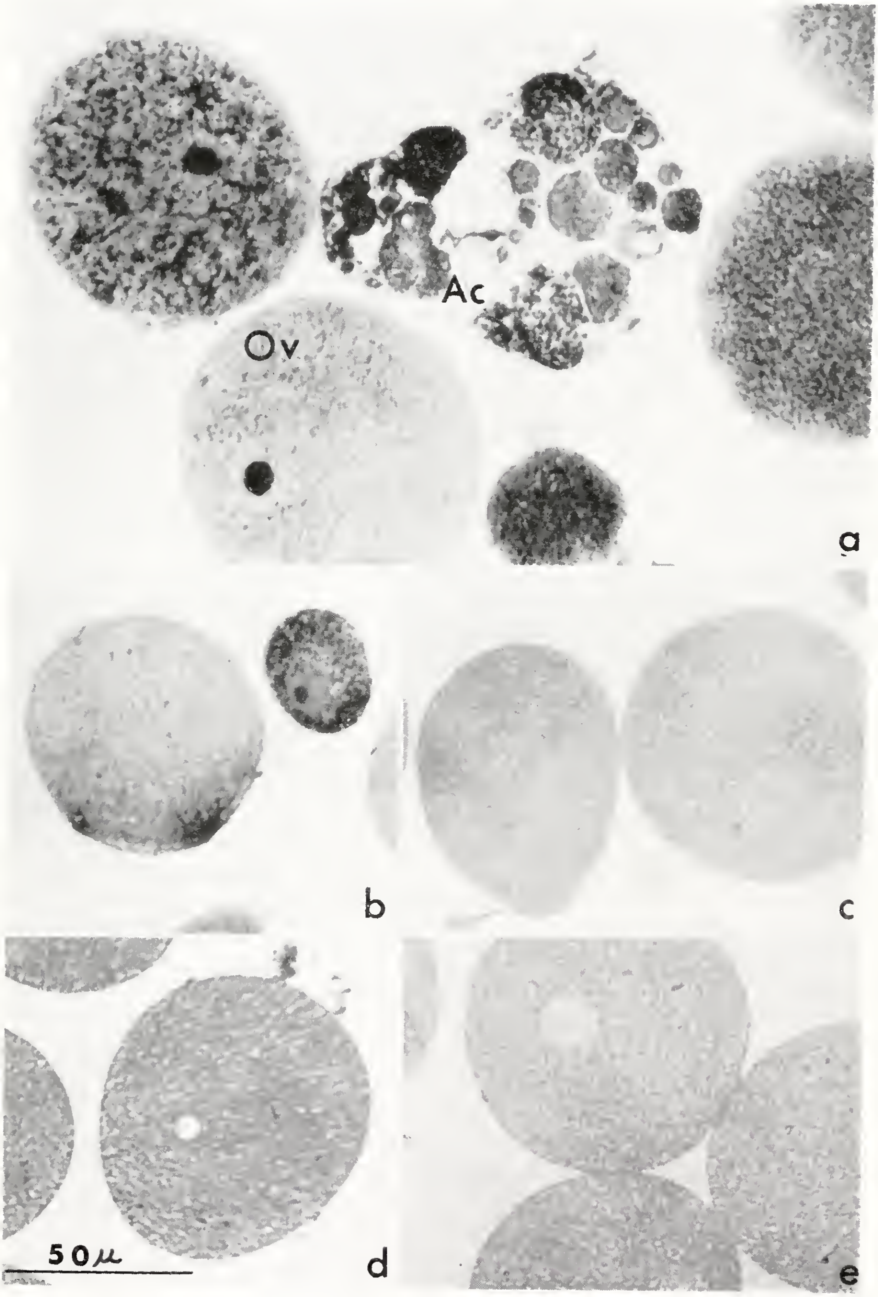

FIGURE 7. Relict ova and mature ova of *A. crassispina* treated by Gomori method: a, accessory cells and relict ova at stage V; b, relict ovum and small oocyte; c, control (without substrate) at stage V; d, mature ova at stage IV; and e, control (without substrate) at stage IV. Ac represents accessory cells; and Ov, ovum.

primary oocytes and become ova. This first period corresponds to the stage when nutrients are stored.

Many observers of the sea urchin gonad have reported on the accessory cells. In one state, they appear almost empty except for a few inconspicuous globules (Chatlynne, 1969). The two species of sea urchins used in this study showed a similar pattern of changing. In particular, the accessory cells around the degenerating ova contain numerous mosaic globules (Fig. 5) which include cortical granule-like structures (Fig. 6). This observation presents an interesting problem in connection with the mode of disposal of the relict ova.

The globules in the accessory cells are stained very strongly by the periodic-acid-Schiff technique, indicating that they include substances which may be of nutritional value for the developing oocytes (Cowden, 1962; Chatlynne, 1969; Bal, 1970). So far there is only indirect evidence that these substances are transferred to the oocytes; *e.g.,* Takashima and Tominaga (1975) found that ^{3}H-D-glucose is present in "nurse cells" three days after injection and only appears in the oocytes after the fourteenth day. Also, a number of investigators have observed that the space occupied by accessory cells decreases as the sensitivity of the oocytes to the PAS reaction becomes stronger, until only empty accessory cells are observed around the fully grown oocytes. The mechanism by which the nutrients are transferred from accessory cells to oocytes has been an object of concern on the part of many investigators. Liebman (1950) observed figures which he interpreted as oocytes phagocytizing accessory cells. Bal (1970) reported that glycogen particles, packed with a protein-like substance, are released into the intercellular spaces. Takashima and Takashima (1965), Tsukahara (1970) and Bal (1970) presented electron microscopical evidence that glycogen particles are taken into the oocytes as pinosomes and used as yolk precursor material.

The manner in which unshed sea urchin gametes are disposed after the end of the breeding season seems to have received only casual attention. Fuji (1960) proposed that "unshed" degenerating ova are absorbed into the follicle cells. Holland and Giese (1965) observed that spermatids are injested by the nutritive phagocytes of the testis early in the reproductive season and again at its end, and assumed that unshed ova also are disposed by a similar process in the ovary. These investigators, however, do not show intermediate stages of relict ova being phagocytized, although they suggest that this might take place. Beig and Cruz-Landim (1975) have investigated in some detail the process by which the residual germ cells are removed from the sea urchin testis and report that spermatids and spermatozoa are phagocytized by "nurse cells" during the interval between reproductive periods. They show electron micrographs in which sperm nuclei, and in some cases flagella, are contained within "digestive vacuoles" *ca.* 10 μm in diameter. Both the size of these vacuoles and the fact that they include left-over gamete organelles suggest a functional parallel with the "mosaic globules" of the sea urchin ovary described in this study. While it is easy to imagine spermatids and spermatozoa being injested by interstitial cells of the testis, the phagocytosis of relict eggs would seem to present a more difficult problem to the accessory cells of the ovary. Several observations made in the course of the present study indicate that autolytic activity may be responsible for the degenerative changes observed in the relict ova. The results of the Gomori test show a significantly stronger acid phosphatase activity in these cells than in normal ova. The number of yolk granules in the degenerating ova is greatly reduced, and their shape is irregular. The cortical layer remains relatively unchanged as compared with normal ova. These morphological observations, and the results of Gomori tests, suggest that lysosomal disruption might occur during the course of degeneration of sea urchin ova. After this stage, "relict" ova might be taken into accessory cells as in the case of spermatids (Holland and Giese, 1965; Beig and Cruz-Landim, 1975). Numerous mosaic globules appear around degenerating ova at stage V, which might indicate some intimate relationship between mosaic globules and degenerating ova. The result that some of the accessory cells and small oocytes showed marked deposits of lead sulfate suggests that these cells degenerate by autolytic processes. Further detailed observations are necessary to make this process completely clear.

Summary

1. Specimens of the sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus,* were collected each month, their ovaries were fixed and embedded in Epon 812, and thick sections were observed with the light microscope.

2. The sea urchin ovary has a clearly defined state in which nutrient globules are produced and stored in accessory cells. Especially in *Hemicentrotus pulcherrimus,* this state continued from May to November. The number of globules in the accessory cells fluctuated with the course of the reproductive cycle.

3. Toward the end of the breeding season, strong acid phosphatase activity was detected in the unshed (relict) ova as they degenerated, and numerous large mosaic globules containing cortical granules appeared in the accessory cells. It is proposed that the cytoplasm of relict ova is disrupted by the activity of their lysosomes.

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CONTROL OF ANTENNAL HAIR ERECTION IN MALE MOSQUITOES

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Thirty years ago Roth (1948) provided conclusive evidence that males of the yellow fever mosquito, *Aedes aegypti,* are sexually attracted to the flight sound of the female. Sound is apparently the only sexual stimulant in this species and males are strongly attracted to and will attempt copulation with any sound source of the appropriate frequency (350–550 Hz). Roth (1948) demonstrated that the male's antennae are the organs by which sound is received. *Aedes aegypti,* like many other species of mosquitoes, has sexually dimorphic antennae. The female antennae are slender and bear rather short setae, whereas those of the male possess 12 equally spaced whorls of very long fibrillae (referred to as *hairs* hereafter), which give the antennae a characteristic bushy appearance. On the basis of extensive experiments, Roth (1948) concluded that the male perceives the female when his long antennal hairs are set into resonant vibration by the sound of the flying female. This vibration is mechanically transmitted to the shaft of the antenna whose motion in turn stimulates the scolopidia of Johnston's organ in the bulbous pedicel of the antennae. Although this phenomenon has only been critically investigated in *Aedes aegypti* (Roth, 1948; Tischner, 1953; Tischner and Schief, 1955; Keppler, 1958; Wishart and Riordan, 1959), a mating response to sound can be demonstrated in males of many other species of mosquitoes, and it is believed that sound is the primary sex attractant in all species whose males possess long antennal hairs (Clements, 1963; Downes, 1969). The mating behavior of species whose males do not possess long antennal hairs, (*e.g., Deinocerites cancer, Opifex fucus, Culiseta inornata*) suggest that these use pheromones for sexual attraction (Kliewer, Miura, Husbands and Hurst, 1966; Provost and Haeger, 1967).

In *Aedes aegypti* and many other species of the subgenus *Stegomyia,* as well as in *Culex pipiens* and *Toxorhynchites brevipalpis,* the long hairs on the mature male's antenna are permanently erect (Roth, 1948 and personal observations). These species are capable of mating at any time of day. This stands in contrast to the situation in many other species of *Aedes, Anopheles, Culex, Mansonia* and *Psorophora,* in which these hairs are closely appressed to the shaft of the antenna during the daytime and become erect only at dawn and dusk coincident with the brief period of swarming and mating activity (Nielsen and Nielsen, 1958, 1962; Nielsen, 1964; Foster and Lea, 1975). In addition, males of species like *An. balabacensis, An. maculatus* and *Ae. vexans* that will not mate under laboratory conditions are not known to erect their antennal hairs in captivity (Dr. George B. Craig, Jr., University of Notre Dame, personal communication; and personal observations).

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Young adult males of *Aedes aegypti*, whose antennal hairs have not yet erected, do not respond to the female flight sound. This suggests that males are incapable of hearing the females when their antennal hairs are recumbent on the shaft of the antenna (Roth, 1948; Wishart and Riordan, 1959). It is therefore reasonable to assume that males of those species which show a daily rhythm of antennal hair erection are not capable of detecting females (and thus incapable of mating) during the daytime when their antennal hairs are recumbent.

The present paper deals with the control of the mechanism that induces antennal hair erection in *Anopheles stephensi* and demonstrates that antennal hair erection is under direct nervous control, disproving the common notion (*e.g.*, Downes, 1969) that changes in blood pressure are the causative event.

MATERIALS AND METHODS

Mosquitoes were reared in a temperature-controlled insectary at 27° C under a 16L:8D photoperiod regime. Adults had continuous access to a dilute sugar solution. All experiments were performed on 4–9 day old males at a room temperature of 23–25° C during a six hour period in the middle of the light phase of the photoperiod.

The saline used in these experiments was that of Lum (1961) adjusted to pH 6.5. All drugs were purchases from SIGMA Chemical Co. with the exception of Phentolamine (gift from Ciba-Geigy Corp.), Terbutaline (gift from Astra Pharmaceutical Products, Inc.), and Salbutamol (gift from Schering Corp.). Experiments on intact males were performed with the animals restrained on their backs on a glass slide covered with a thin layer of petroleum jelly. Injections were done *via* a glass capillary drawn to a fine tip on a micropipette puller. About 0.10–0.15 μl of the experimental solution was injected into the thorax of each male. Isolated antennae were prepared by grasping the male's proboscis with forceps and cutting just below the pedicel of the antennae with iridectomy scissors. In this way

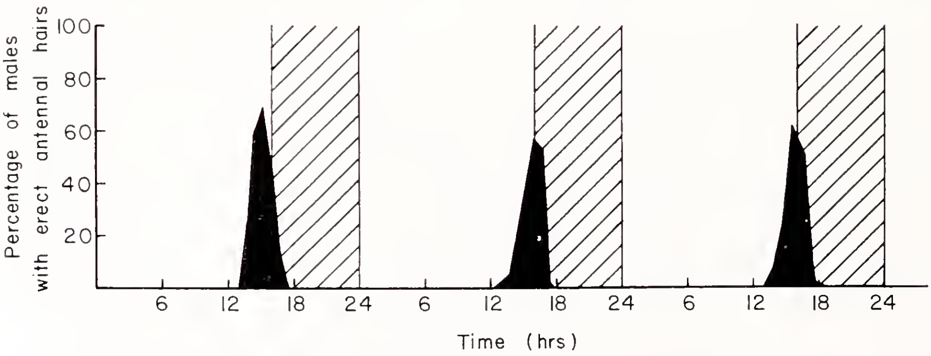

FIGURE 1. Rhythm of antennal hair erection in males of *Anopheles stephensi* under a 16L:8D photoperiod with abrupt light-dark transitions. Shaded areas represent periods of darkness. Data represent the percentage of males with erect antennae during three consecutive photoperiodic cycles in a population of 300 males held in gallon-size cages. Males begin to erect their antennal hairs about 3 hours before lights-off. All males have recumbent hair again 6 hours later.

antennae, palps and proboscis came off as a unit, sometimes with a small portion of compound eye attached. The proboscis and palps thus served as a convenient handle to transfer antennal sets without damage to the delicate antennae. Control experiments showed that the presence of proboscis, palps and the pedicel of the antenna in no way affected the response of isolated antennae. Antennal sets were floated, base down, on a drop of saline resting on a siliconized glass slide. This slide was placed in a petri dish lined with moist filter paper to prevent dessication of the preparations. In the absence of further stimulation the antennal hairs remained recumbent for at least four hours in such preparations. A scoring system was developed for assaying the effectiveness of various chemical stimuli in inducing antennal hair erection. An antenna with all hairs recumbent was given a score of 0, while an antennae with all hairs erect received a score of 10. Intermediate scores (1–9) were obtained by estimating the proportion of hair whorls that had been erected (see Figure 3). When the mean score of 10–20 antennal sets was taken, the response to a given drug solution was found to be quite reproducible. All experiments reported below were performed with males of *Anopheles stephensi.*

RESULTS

The antennal hair erection rhythm of *Anopheles stephensi* males was studied under a 16L:8D photoperiod with abrupt day-night transitions. Animals used in this experiment were maintained in gallon-size cages in the absence of females. Figure 1 shows that under these conditions males began to erect their antennal hairs in anticipation of the onset of darkness, and the percentage of males with erect antennal hairs was already on the decline at lights-off. This behavior indicates that the hair erection rhythm is governed by a circadian clock and is not merely a response to darkness. In fact, in *An. stephensi,* antennal hair erection *cannot* be induced at any time during the photophase either by sudden darkness or by gradual light dimming. Under the conditions of this experiment, there was no hair erection at or about the lights-on signal. Thus, antennal hairs were recumbent at all times except for a 3–4 hour period immediately preceding the onset of darkness. A maximum of 60% of the males in the experimental population had erect hairs at any one time. It is not known at present whether this reflects an asynchrony among members of the population or whether only 60% of the individuals are capable of responding during any one cycle. Preliminary evidence indicates that the presence of females does not affect the hair erection rhythm of the males.

In larger cages (8 ft^3) it was possible to find a few males (less than 1% of the population) with erect antennal hairs at any time of day. These males actively copulated with flying females. This stands in contrast to the situation in gallon-size cages where antennal hair erection *and* mating were sharply restricted to the period indicated in Figure 1.

Response of intact males to injected drugs

Possible nervous involvement in the control of antennal hair erection was investigated by injecting a number of known and putative neurotransmitters and other pharmacological agents into intact males. Rapid erection of antennal hairs occurred upon injection of various catecholamines and other sympathomimetic agents. The lowest concentration of these substances that caused erection of all hairs within five minutes and the persistence of erection for at least twenty minutes were: DL-synephrine, 0.1 mM; L-epinephrine, 0.2 mM; DL-octopamine, 1 mM; L-phenylephrine, 2 mM; DL-norepinephrine, 4 mM; and dopamine, 8 mM. The rather high concentrations required are probably due to rapid metabolism of the drugs making it unlikely that differences in activity of the various isomers could be detected. Injections of up to 30 mM acetylcholine, atropine, physostigmine, neostigmine, pilocarpine, γ-aminobutyric acid or 5-hydroxytryptamine were without effect. Injection of all sympathomimetics induced simultaneous erection of all hair whorls 2–3 min after injection except for phenylephrine which first induced erection of the proximal whorls followed progressively by the more distal ones over a 3 minute period. The former response can be explained on the basis of a rapid distribution of the drugs throughout the antenna by the circulatory system and compares favorably with the normal (photoperiodically stimulated) erection pattern in which all hairs became erect simultaneously in the course of about 5 min. The response to phenylephrine is not readily interpreted at present. Injections of synephrine, epinephrine and occasionally octopamine also caused flexion of the distal palp segments identical to that observed during spontaneous antennal hair erection at dusk (Figure 2a, b). Flexed palps are characteristic of swarming males, but no function has yet been elucidated for this condition. Finally, intact males also erected their antennal hairs upon anesthesia with either chloroform or CO_2. Depth of the anesthesia, judged by the time required for recovery, determined the degree to which the hairs were erected, as well as the duration of erection.

Response of isolated antennae to sympathomimetic drugs

The results presented above indicate that blood-borne sympathomimetic agents can induce antennal hair erection. To determine whether these compounds acted

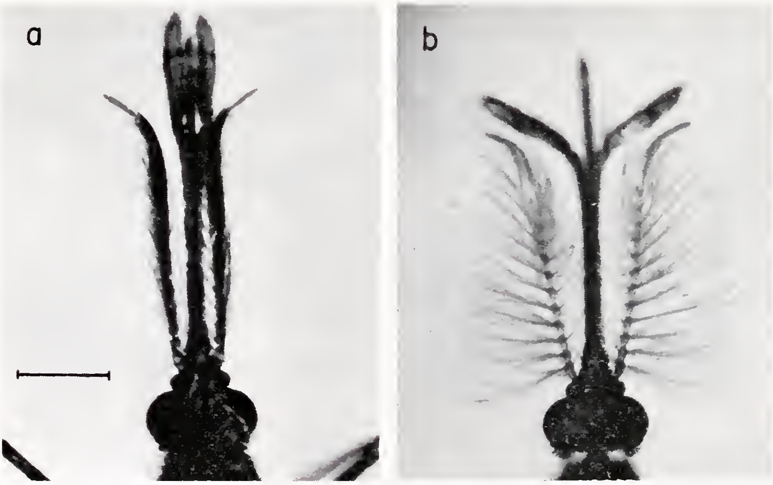

FIGURE 2. a. Head of male *Anopheles stephensi* with recumbent antennal hairs. This is the characteristic configuration during most of the day; and b, male with erect antennal hairs and flexed palp tips about one hour prior to the onset of darkness. Only males with erect antennal hairs can detect a female. Bar is 0.5 mm.

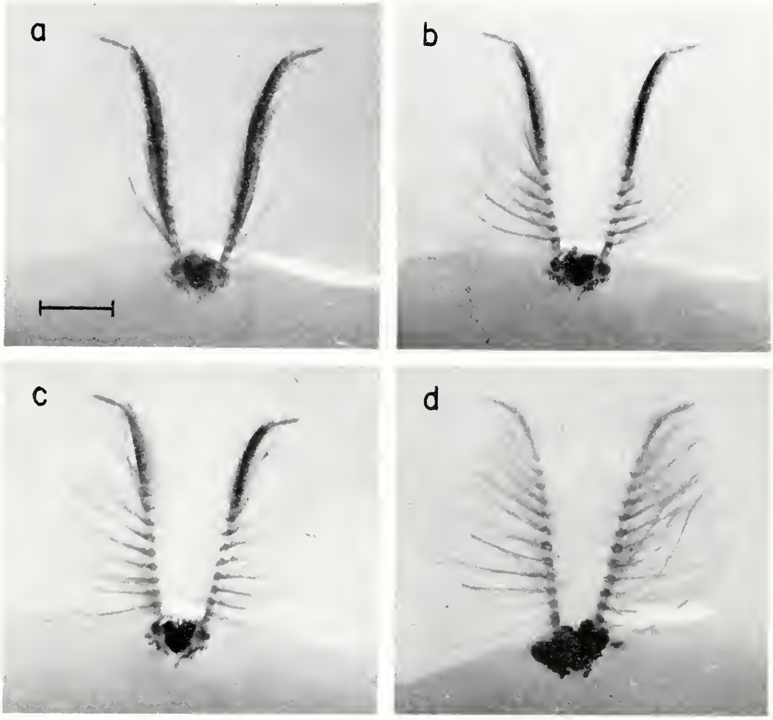

FIGURE 3. Sequence of hair erection in isolated antennae floating on a drop of saline containing 0.5 mM synephrine: a, one minute after initiation of experiment; b, three minutes; c, five minutes; and d, twelve minutes. The response of these antennae is scored as 0, 4, 6 and 10, respectively, according to the scoring system described in the text. Bar is 0.5 mm.

directly on the effectors in the antennae, isolated antennae were floated, base-down, on drops of saline containing appropriate concentrations (1–10 mM) of a sympathomimetic agent. Under these conditions, the proximal hairs became erect in about 2 to 3 minutes and were gradually followed by the more distal hairs (Figure 3). It is likely that this sequence of hair erection followed the diffusion of the exogenous drug up the antennal shaft. These results show that blood-borne sympathomimetics act directly on the effectors for hair erection in the antennae. Flexion of the distal palp segments never occurred in these isolated preparations. This suggests that the palps responded to a secondary stimulus (possibly arising in the central nervous system) and not to the drug itself.

Since isolated antennae can erect their hairs normally it is clear that changes in blood pressure are not involved in this response. Isolated antennae provided a convenient and reliable assay for studying the effects of various stimuli on antennal hair erection. The response was quantified by determining the proportion of hair whorls on an antenna that had become erect at various times after exposure to the drug solution (see Methods and Fig. 3). Figure 4 illustrates the time course of the response of isolated antennae to various concentrations of synephrine, epinephrine and octopamine. The point at which each curve reached a plateau presumably

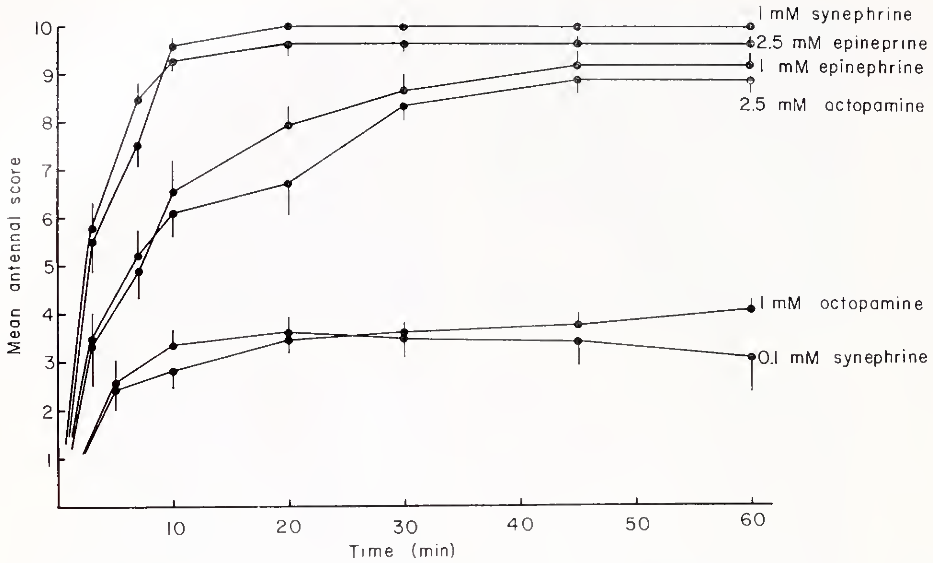

FIGURE 4. Response of isolated antennae to various sympathomimetic agents. Each curve represents the mean of two replicates of 24 antennae each. Scoring system for hair erection reflects the proportion of hair whorls that had become erect at a given time (*cf.*, Figure 3). Bars indicate standard errors.

reflects the achievement of a suprathreshold concentration of the amine throughout the antennae. Some antennae were slightly damaged in setting up the experiment and failed to erect all hairs. This accounts for plateaus somewhat below the maximum mean score. More significantly, the curves for 0.1 mM synephrine and 1 mM octopamine reached a plateau when only the proximal 30% of the hairs had become erect. This stands in contrast to the findings on intact animals where these concentrations of drugs induced erection of *all* antennal hairs. In the latter case, swift metabolism of the drugs was evident by the short persistence of hair erection (20–30 minutes). If metabolism of these drugs also occurred in isolated antennae, then the failure of the more distal hairs to become erect was probably due to the fact that a suprathreshold concentration could not be achieved beyond a point along the antennal shaft where metabolism of the drug balanced its arrival by diffusion. This point shifts progressively more distally with increasing concentrations of drug.

Effect of picrotoxin

Picrotoxin is a central nervous system stimulant that acts by blocking the activity of γ-aminobutyric acid, an inhibitory neurotransmitter in vertebrates and invertebrates. Injections of 1 mM picrotoxin into intact males induced rapid erection of all antennal hairs that persisted for several hours (threshold dose, as defined above, was 0.1 mM). In contrast to the action of sympathomimetic agents, picrotoxin did not induce hair erection in isolated antennae even at a 30 mM concentration. In order to localize the site of action of picrotoxin, progressively larger portions of head and thorax were removed with the antennae and floated with the cut surface onto a saline drop containing 1 mM picrotoxin. Figure 5 shows that the presence of the head and the anterior half of the thorax were required for picrotoxin to stimulate hair erection. It is unlikely that the inactivity of picrotoxin in isolated antennae was due to a failure of this substance to penetrate the antennae, because fluorescence microscopy showed that fluorescein, added to the picrotoxin-saline, readily diffused up the antennal shaft. Conversely, when isolated head-thorax preparations were treated with a picrotoxin solution that also contained fluorescein, no dye could be detected in the antennae for at least 20 minutes after the hairs became erect. This observation indicated that picrotoxin probably had not penetrated the antennae at the time that the hairs became erect.

These results suggest that picrotoxin acted on a center in the thorax that, in turn, was responsible for inducing erection of the antennal hairs. When males that had been injected with picrotoxin were decapitated, the antennal hairs on the isolated heads assumed their recumbent position within 3 min, while on intact control animals, they remained erect for several more hours. Thus, the thoracic center is also required for the maintenance of hair erection. Destruction or bisection of the brain completely abolished the response to picrotoxin in otherwise intact animals. This observation indicated that the brain is needed in this response, although its precise role and the nature of its interaction with the thoracic center are not clear at present.

Pathway of control for the erectile mechanism

There are two ways in which the thoracic center that is activated by picrotoxin could control the erection of antennal hairs. It may cause the release of a humoral substance from a site outside the antennae, which in turn is carried to the effectors by the bloodstream. Alternatively, it may cause a nervous signal to be sent directly

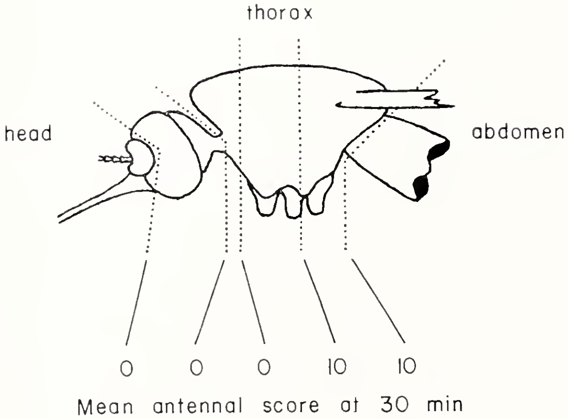

FIGURE 5. Response of various sections of male mosquitoes to picrotoxin. Cuts were made at the level of the dotted lines and the anterior portions floated with their cut ends on a drop of saline containing 1 mM picrotoxin. Numbers represent the degree to which antennal hairs had erected 30 minutes after exposure to picrotoxin. Each operation was replicated 35–40 times. It appears that the head as well as a portion of the thorax are required in the response to picrotoxin.

to the effectors in the antennae. To distinguish between these alternatives nervous input into the right-hand antenna was abolished in 60 animals by cutting the antennal nerve with a hooked needle through an aperture made by cutting off the proboscis and palps at their base. In 51 of these animals only the hairs of the left-hand antenna became erect upon subsequent injection of a solution of picrotoxin (1 mg/ml) containing a small amount of fluorescein. In two animals a few of the more distal hair whorls became erect, and all hairs of the right-hand antenna erected in the remaining seven. Upon dissection, it appeared that the antennal nerve of the latter animals was not significantly damaged. In order to ensure that the operation had not interfered with the circulation of hemolymph into the right-hand antennae, all antennal pairs were examined under a fluorescence microscope about five minutes after the hairs on the left-hand antenna had fully erected. The distribution of fluorescein in both antennae appeared identical in most cases. In several instances the antenna on the operated side contained more dye than the one on the intact side. In addition, twelve males that had failed to erect the hairs on their right-hand antennae were injected with a solution of synephrine (0.03 μl, 1 mM) five to ten minutes after the hair on the left-hand antenna had become erect. The synephrine injection induced erection of all hairs on the right-hand antenna within two minutes. It is clear from these experiments that circulation into the antenna on the operated side was not significantly affected, nor was the erectile mechanism of the antenna organically damaged. The results of these experiments indicate that the erection of antennal hairs is not controlled by a blood-borne factor. The most likely alternative is that hairs are controlled by direct nervous input into the antennae.

Electrical stimulation of antennae

Further evidence for nervous control of the erectile mechanism of antennal hairs was provided by experiments in which antennae, *in situ* or isolated, were electrically stimulated. Square wave stimuli of 150–200 mV amplitude and 10 msec duration at a frequency of 20 cps were applied to intact animals by inserting one electrode into the thorax and the other into the cut end of one antenna. Antennal hairs became erect within 30 seconds of stimulation. Erection persisted as long as stimulation was applied and subsided about one minute after cessation of stimulation. Hairs on the unstimulated antenna of these experimental animals never showed any evidence of erection. Electrical stimulation also induced hair erection in isolated antennae. In these experiments single antennae were arched between two drops of saline resting on a siliconized microscope slide so that only the two ends of the antenna were immersed. The stimulating electrodes were then inserted into these saline drops. Hair erection followed shortly upon initiation of stimulation though somewhat higher voltages (300–450 mV) were required to evoke a response in these isolated antennae than were necessary in antennae *in situ*. This difference in apparent sensitivity was probably due to a greater separation of electrodes in the latter experiment.

Evidence for the involvement of antennal motor neurones in the response to electrical stimulation was obtained from two different experiments. In the first set, males were injected with tetrodotoxin (1 μg/μl in saline), a substance that specifically blocks the sodium current in axons, rendering them incapable of conducting action potentials. Four to six hours later their antennae (*in situ* or isolated) were electrically stimulated as described above. Only one instance of hair erection was found in ten animals treated this way, and in that single instance the antennal hairs became only partially erect. When isolated antennae of these tetrodotoxin-treated males were subsequently floated on drops of saline containing 5 mM octopamine, all hairs erected normally. Thus, tetrodotoxin did not affect the effector mechanism itself but probably prevented response of motor axons in the antenna.

In the second set of experiments, isolated antennae were floated on drops of saline containing an elevated concentration of potassium ions (60 mM; isotonicity of the saline was conserved by a proportional decrease in the concentration of NaCl). Such high concentrations of potassium are known to cause depolarization of axons and release of neurohormones or neurotransmitters in insects (Maddrell and Gee, 1974; Usherwood, 1974). Antennal hairs began to erect within one minute after exposure to high-potassium saline, and all hairs were fully erect ten minutes later. The hairs remained erect for 20–30 minutes and then gradually returned to their recumbent position. All hairs were fully recumbent by 1.5–2 hours later. After all the hairs had become recumbent, the antennae were electrically stimulated as described above. Not a single one of 12 antennae tested in this way erected its hairs. However, all of these antennae erected their hairs normally when they were subsequently placed on saline drops containing 5 mM octopamine. It is thus clear that the effector mechanism for hair erection was not affected by the high-potassium treatment. It seems reasonable to conclude from these results that the loss of response in antennae exposed to high-potassium saline was due to local depletion of neurotransmitter at the motor axon-effector synapse and possibly also due to the fact that action potentials could no longer be generated in the chronically depolarized axons.

Attempts to demonstrate Ca^{++}-dependence of antennal hair erection have failed so far, probably due to the fact that there are numerous hemolymph cavities within the antenna that are not readily flushed out [see Risler (1953) for a description of the anatomy of a mosquito antenna] and could serve to sequester calcium ions.

Evidence for second messenger involvement in the response to sympathomimetics

There is increasing, though still circumstantial, evidence that catecholamines and other sympathomimetic agents induce the synthesis of cyclic adenosine monophosphate (cAMP) in their target cells and that the increased levels of cAMP mediate the cell-specific reactions to these substances. In order to determine a possible role of cyclic nucleotides in mediating antennal hair erection, isolated antennae were incubated on saline drops containing cAMP or one of its derivatives or cGMP, in the presence or absence of theophylline. Figure 6 illustrates the results of these experiments and shows that, in the presence of theophylline, 8-bromo-cAMP was highly effective in stimulating hair erection, whereas this compound was inactive when supplied in the absence of theophylline. Theophylline alone produced an intermediate response. Cyclic AMP and dibutyryl-cAMP did not enhance the response to theophylline. Presumably these compounds were unable to penetrate the effector cell membrane.

The response to 3mM cyclic GMP in the presence of theophylline was identical to the response to 8-bromo-cAMP. Cyclic GMP did not evoke antennal hair erection in the absence of theophylline.

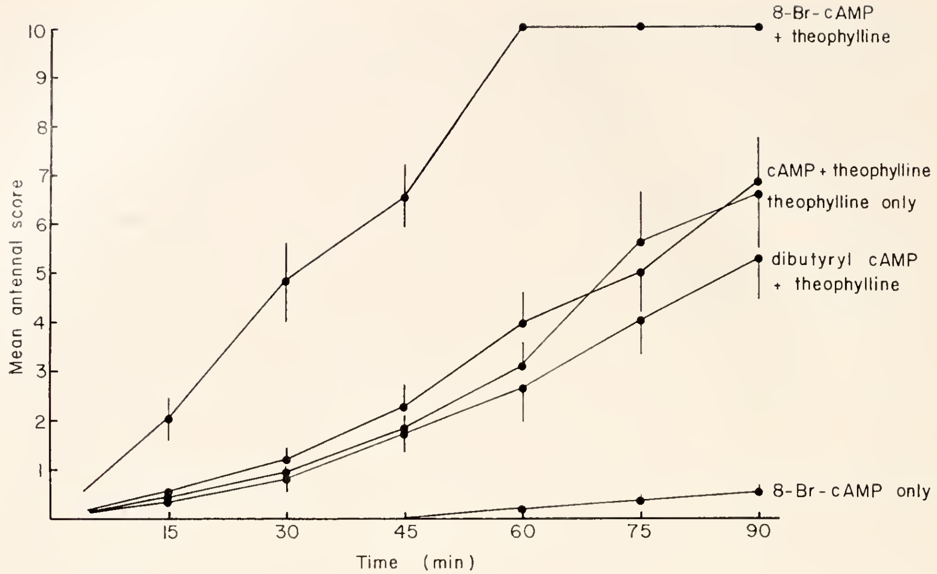

FIGURE 6. Effect of cyclic nucleotides and theophylline on hair erection in isolated antennae. Cyclic nucleotide concentrations were 3 mM; theophylline, 8 mM throughout. Each curve represents the mean of 6 replicates and a total of 90–124 antennae. Bars indicate standard errors.

The experiments described above show that cyclic nucleotides can mimic the action of sympathomimetic agents. Although a role for cyclic nucleotides in mediating the action of the neurotransmitter is indicated, further proof of this will have to await determination of a rise in their titer after stimulation.

Nature of the adrenergic receptor

In view of the specificity of hair erection for exogenous sympathomimetic agents, it was of some interest to determine the nature of the cell surface receptor. Groups of males were pretreated with an injection of 10 mM phentolamine (a specific α-adrenergic blocking agent) or 10 mM propranolol (a β-adrenergic blocking agent). After 45 minutes, antennal sets were removed and floated on saline drops containing either 1 mM epinephrine or 1.5 mM octopamine with 10 mM of one or the other blocking agent. Inhibition of the response to octopamine and epinephrine occurred in the presence of phentolamine, but propranolol had not effect whatsoever (Table I). These results show that the cell receptor (presumably on the effector cells for antennal hair erection) resembles the vertebrate α-adrenergic receptor. The specificity of the receptor was examined further by injecting intact males or treating isolated antennae with terbutaline or salbutamol, sympathomimetic agents having exclusively β-adrenergic activity. These compounds were totally ineffective in inducing antennal hair erection in concentrations up to 30 mM. When phentolamine-blocked isolated antennae were treated with 8-bromo-cAMP plus theophylline, antennal hair erection occurred normally showing blockage was not due to a nonspecific toxic effect of this drug (Table I).

Discussion

The evidence presented above strongly suggests that erection of the antennal hairs of males of *Anopheles stephensi* is under direct nervous control. The finding that partial severance of the antennal nerve can abolish erection of the proximal hairs, while leaving the distal ones under central control, indicates that motor neurons must extend up the entire length of the antennal flagellum. This is a surprising finding since motor neurons have been assumed to be absent from antennae of pterygote insects.

Knowledge of the internal anatomy of mosquito antennae is limited to the work of Risler (1953, 1955), whose findings and descriptions offer no clue as to the effector organ of hair erection. A few cells occur at the base of each antennal hair. These were believed to be sensory neurons (Risler, 1953), but recent evidence indi-

Table I

Effects of sympathomimetic drugs and adrenergic blocking agents on hair erection in isolated antennae of Anopheles stephensi *males.*

Compound	Conc (mM)	Agonistic activity	Blocking activity	Mean score at 60 min*
Effect of adrenergic blocking agents				
Epinephrine	1	α, β	—	9.8
Octopamine	1.5	α, ?	—	5.8
Epinephrine	1	α, β	—	9.1
plus				
Propranolol	10	—	β	
Octopamine	1.5	α, ?	—	6.0
plus				
Propranolol	10	—	β	
Epinephrine	1	α, β	—	0.0
plus				
Phentolamine	10	—	α	
Octopamine	1.5	α, ?	—	0.6
plus				
Phentolamine	10	—	α	
Specific α- and β-adrenergic drugs				
Synephrine	5	α	—	10
Phenylephrine	10	α	—	10
Norepinephrine	10	α	—	9.8
Dopamine	10	α	—	5.8
Terbutaline	30	β	—	0.0
Salbutamol	30	β	—	0.0
α-blockage bypass with cyclic nucleotide				
Phentolamine	10	—	α	9.2
8-bromo-cAMP	8	—	—	
Theophylline	10	—	—	

* Each score is the mean response of 3 replicates of 18–24 antennae each.

cates that at least some of these cells are part of the effector mechanism for antennal hair erection (Nijhout and Sheffield, in preparation). Definitive proof of synaptic transmission at the effector site will probably prove exceedingly difficult, if not impossible, to obtain. The antennae of this species are only 20 μm in diameter and direct access to the antennal nerve is hindered by the fact that it is encased in two layers of the antennal skeleton.

The only pharmacological agents capable of eliciting hair erection in isolated antennae are sympathomimetic agents and, of these, only those that possess α-adrenergic activity. Since the response (of isolated antennae) to these drugs is blocked by phentolamine but not by propranolol (Table I), it appears that an α-adrenergic-like receptor occurs in the membrane of the effector cells. It is therefore possible that a catecholamine or a phenolic amine is the natural neurotransmitter that acts on the effector cells in the antennae. The relative potency of the sympathomimetics used is identical to that found in the firefly lantern (Carlson, 1968). Robertson and Carlson (1976) have suggested that octopamine is the natural neurotransmitter in that system. However, other catecholamines, particularly dopamine, have been implicated as neurotransmitters in nonmuscular effector organs of insects such as the salivary glands (Bland, House, Ginsberg and Lazlo, 1973; Robertson, 1975). Although dopamine has a relatively low activity in induction of hair erection, its possible involvement in this process cannot be ruled out at present. It should be noted here that it has proven impossible to demonstrate the presence of catecholamines in antennae by means of the Falck-Hillarp catecholamine-fluorescence technique due to high background fluorescence in these organs. Thus, final resolution of the identity of the neurotransmitter will be possible only after detailed biochemical studies.

The results of experiments using picrotoxin indicate that the nervous system in the head is not sufficient to either initiate or maintain erection of the antennal hairs. Rather, a portion of the thoracic nervous system is required for picrotoxin-stimulated hair erection. This is a puzzling finding, since it would be reasonable to assume exclusive cerebral control over this event. The data presented above do not allow any conclusions about the relative roles of the cerebral and thoracic nervous systems in hair erection. Any further speculation on the central control of hair erection will have to await more detailed neurophysiological investigations.

I wish to thank Drs. Louis H. Miller and Robert W. Gwadz for critical reading of the manuscript.

SUMMARY

The long fibrillae, or hairs, on the antennae of male mosquitoes are essential for the detection of the female flight sound, the sole sexual attractant. In *Anopheles stephensi,* as in many other genera and species, these long hairs are recumbent on the shaft of antenna during the daytime and become briefly erect at dusk, coincident with swarming and mating activity. Evidence is presented that erection of the antennal hairs is under direct nervous control. Isolated antennae can be induced to erect their hairs only in the presence of α-adrenergic agonists, and this response is blocked in the presence of the α-adrenergic blocking drug, phentolamine. Thus, a cell surface receptor, resembling the vertebrate α-adrenergic receptor, probably

mediates the response to the natural neurotransmitter. The action of α-adrenergic drugs is mimicked by cyclic nucleotides and also by theophylline.

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ASEXUAL REPRODUCTION AND GENETIC POPULATION STRUCTURE IN THE COLONIZING SEA ANEMONE *HALIPLANELLA LUCIAE*

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The acontiate sea anemone *Haliplanella luciae* (Verrill), since its presumed origin on the Pacific coast of Asia (Stephenson, 1935), has become distributed throughout the Northern Hemisphere. Populations are known to occur in Japan, on the west coast of North America and on the East Coast from Maine to Florida, in the Gulf of Mexico, in the British Isles and Western Europe, in the Mediterranean Sea and Suez Canal, and in Malaysia. Detailed zoogeographic records and historical accounts of the spread of *H. luciae* can be found in Parker (1902), Uchida (1932), Stephenson (1935), Carlgren and Hedgpeth (1952), and Hand (1955b). The dispersal of the species has been effected through attachment to oysters shipped commercially (Verrill, 1898), ship bottoms (Stephenson, 1935), and seaweed (Williams, 1973a).

Local populations of *Haliplanella* are often ephemeral, showing a tendency to appear suddenly, flourish for a time, and then to disappear abruptly (see Stephenson, 1953). A noteworthy aspect of the biology of this actinian is its eurytolerant nature, individual specimens surviving intertidal exposure to high summer temperatures, encasement in ice in winter, and exposure to drastically reduced salinities (Verrill, 1898; Hausman, 1919; Miyawaki, 1951; Kiener, 1971; Shick, 1976). Populations of *H. luciae* thus exhibit the temporal and physiological characteristics of colonizing species.

Uchida (1932) has described four distinct morphs of the species based on color and stripe pattern, although other records and unpublished observations suggest that variation may be more continuous. Local populations may be exclusively of one morph or (especially in locations geographically close to the species' center of origin) a mixture of several (Ômori, 1895; Uchida, 1932; Hand, 1955b; Williams, 1973b). A variety of synonymns has been applied to the species. Furthermore, as might be expected in such a widespread species, physiological races (*sensu* Stauber, 1950) have been described in isolated localities (Williams, 1973b; Shick, 1976). For example, individuals in the population studied by Sassaman and Mangum (1970) in Virginia typically show reduced metabolic activity and encystment in mucus at 10° C and below, while individuals from Maine are cold-adapted, remaining expanded and active at 0° C and below (Shick, 1976). According to the acclimation criteria of Prosser (1957), the demonstration of positive thermal compensation of metabolic rate in the Maine population (Shick, 1976) and of inverse thermal acclimation in the Virginia population (Sassaman and Mangum, 1970), would indicate that the two populations represent genetically determined physiological races.

Despite the current proliferation of accounts of genetic variation in natural populations, the genetic structure of sea anemone populations has received little attention. The few exceptions to this generalization are the studies of Francis (1973a, b; 1976), Cain (1974), Dunn (1975), Ottaway and Kirby (1975), and Hoffmann (1976). The theoretical foundations for genetic investigations of sea anemones have been laid by students of the genetics of plants, sessile organisms which, like anemones, possess alternative modes of sexual and asexual reproduction.

In a preliminary paper, Shick (1976) presents evidence that the large population of *Haliplanella* at Blue Hill Falls, Maine, is a single clone: all specimens are males; reproduction by longitudinal fission is commonly observed; and fixed heterozygosity at a "tetrazolium oxidase" locus exists. Using the criterion of sex, a monoclonal structure of the female population at Plymouth, England, and the male population at Wells, Norfolk, England (R. B. Williams, personal communication), as well as of the female population in Selangor, Malaysia (D. F. Dunn, California Academy of Sciences, personal communication), may be inferred. The added criterion of electrophoretic screening of isolated populations was employed in this study to investigate the generality of this phenomenon in the species.

Finally, to clarify the significance of asexual reproduction in colonizing new habitats and in maintaining locally adapted populations, population structure and geographic distribution of *Diadumene leucolena* Verrill was examined. This actinian, which is a member of the same subtribe (Acontiaria) as *H. luciae* (which itself was formerly placed in the genus *Diadumene*), is ecologically similar to and often sympatric with *Haliplanella,* but is strictly sexual in its reproduction.

MATERIALS AND METHODS

Samples of *Haliplanella luciae* were collected from four sites on the United States Atlantic coast. Physical data for the habitat of the type 1 (12 orange stripes on a greenish-brown column) anemones from Blue Hill Falls, Maine (BHF), have been presented elsewhere (Shick, 1976). A population of type 3 (48 paired white stripes on a green column) individuals from the Barnstable town dock, Massachusetts (BTD), was sampled in June and August, 1976. Types 1 and 2 (nonstriped) *H. luciae* from the Florida State University Marine Laboratory at Turkey Point, Florida (TP), were provided by L. L. Minasian, Jr. in October, 1976.

At Charlestown Salt Pond, Rhode Island (CSP), all four of Uchida's (1932) morphological types are present. In addition to those types already described, three type 4 (combining characters of types 1 and 3) individuals were also seen, and two of these specimens were collected for electrophoretic analysis. Type 1 individuals were likewise very rare at this site on both collecting dates (April and June, 1976), while types 2 and 3 were extremely abundant.

Specimens of *Diadumene leucolena* were collected at Narrow River, Rhode Island, by W. R. Ellington in December, 1975, and by us in April and June, 1976. Narrow River presents a true estuarine situation, with salinities averaging 8 to 10‰ at low tide (Ellington, University of Vermont, personal communication), which is below the critical limit for acclimation in *H. luciae* (Miyawaki, 1951; Shick, 1976), but within the zone of tolerance of the more euryhaline *D. leucolena* (Pierce and Minasian, 1974).

In preparation for electrophoresis, whole anemones were homogenized in two volumes of 0.1 M phosphate buffer, pH 7.0, and centrifuged at 7000 × g for 20 minutes at 0°C. Horizontal starch gel electrophoresis of the supernatant was performed using 12.6% (W/V) gels. At least one individual from the BHF clone was included as an internal standard on each gel. The enzymes and their abbreviations, buffer systems, and stains are listed in Table I. All enzymes were electrophoresed in three buffer systems; those given are those which produced the best resolution. To complement the electrophoretic studies, the sex of most individual anemones was ascertained by microscopic examination of either fresh gonadal smears or of Bouin's-fixed and sectioned specimens.

RESULTS

The variable and invariable enzymes, and the populations of *Haliplanella* in which they were examined, are listed in Table I. Only four enzymes were found to be variable, although leucine aminopeptidase (buffer II, stain B) appeared to be, but resolution was usually too poor for scoring. All observed allozyme phenotypes are shown in Figure 1. Alleles at each locus are designated alphabetically according to electrophoretic mobility of their enzyme products, the fastest-moving band being designated "A." By convention, the observed phenotypes are assumed to reflect the genotypes of individual specimens.

The genotype of each population is shown in Table II. For any given population, the numbers in each column, besides representing the number of individuals scored for that enzyme, also indicate the genotype at that locus. For example,

TABLE I

Enzymes examined electrophoretically in populations of Haliplanella luciae. *Population abbreviations are given in text.*

	Buffer	Stain	Population examined
Variable enzymes			
Hexokinase (HK)	II	A	All
Isocitrate dehydrogenase (IDH)	I, III	B	All
Phosphoglucose isomerase (PGI)	II	B	All
"Tetrazolium oxidase" (TO)	III	C	All
Invariable enzymes			
Esterase	II	A	BHF
Fumarase	IV	A	BHF
Glutamate oxaloacetate transaminase	III	B	All
Malate dehydrogenase	I, IV	B	BHF, CSP Types 2, 3
Malic enzyme	I, IV	B	BHF
Phosphoglucomutase	II	B	BHF
Xanthine dehydrogenase	IV	B	BHF, CSP Types 2, 3

Buffers
I. Tris-citrate, pH 7.0 (Shaw and Prasad, 1970)
II. Lithium hydroxide, pH 8.1 (Ridgway, Sherburne and Lewis, 1970)
III. Tris-borate, pH 8.6 (Markert and Faulhaber, 1965)
IV. Tris-citrate, pH 6.3 (Selander, Smith, Yang, Johnson and Gentry, 1971)

Stains
A. Modified from Shaw and Prasad (1970)
B. Modified from Selander *et al.* (1971)
C. Modified from Brewer (1970)

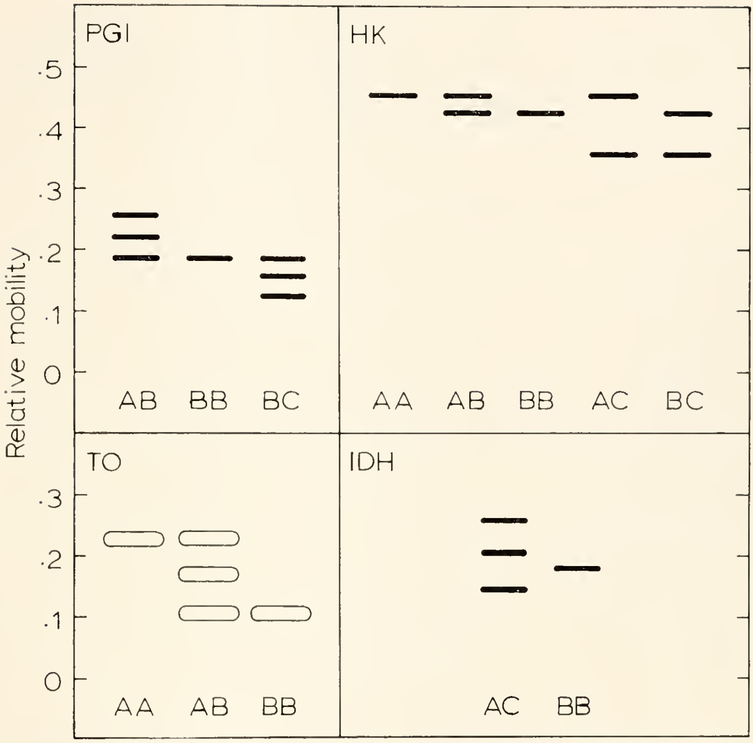

FIGURE 1. All phenotypic classes of allozymes at variable loci observed among populations of *Haliplanella luciae*. Mobilities are relative to bromphenol blue dye front. Anode is at top.

the genotype of all Blue Hill Falls individuals is: PGI- BB; TO- AB; IDH- BB; HK- BB, where 67 specimens were scored for PGI, 58 for TO, 31 for IDH, and 9 for HK. The numbers are different because some gels could not be reliably scored for every individual, and because some of the enzymes were found to be variable after the electrophoretic screening had begun.

All examined populations of *Haliplanella* appear to reproduce strictly asexually. In most cases this can be concluded from the incidence of fixed heterozygosity at one or more of the enzyme loci. In the cases of presumptive clone 1 of the type 1 anemones and of the type 4 specimens from Turkey Point, which are both homozygous at all loci, strict asexuality may be inferred from the sterile condition of the mesenteries. Although the possibility that these individuals may be sexually active during other seasons cannot be ruled out, their extremely small size would indicate that they are not sexually mature: there is an apparent body size threshold for gonadal maturation (D. F. Dunn, personal communication), and our casual observations of BHF individuals support this.

In the sexually reproducing *Diadumene leucolena*, only phosphoglucose isomerase (PGI) was found to be both variable and reliably scored. Results are presented in Table III. These data are pooled for the three collecting dates [G-test of independence (Sokal and Rohlf, 1969), $G = 2.034$, d.f. $= 2$, $0.5 > P > 0.1$],

TABLE II

Numbers of each genotype observed at four polymorphic enzyme loci in clonal Haliplanella luciae *from four Atlantic coast locations. Asterisks denote presumptive clones, which were homozygous at all loci examined.*

	PGI			TO			IDH		HK				
	AB	BB	BC	AA	AB	BB	AC	BB	AA	AB	BB	AC	BC
Blue Hill Falls, Maine													
Type 1 (male)	—	67	—	—	58	—	—	31	—	—	9	—	—
Barnstable town dock, Massachusetts													
Type 3 (sterile)	40	—	—	—	—	40	—	40	—	—	—	—	40
Charlestown Salt Pond, Rhode Island													
Type 1, clone #1 (sex not determined)	—	—	4	—	—	3	—	2	—	—	—	—	—
*Type 1, clone #2 (sex not determined)	—	4	—	3	—	—	—	—	—	—	—	—	—
Type 2 (male)	—	—	47	—	43	—	36	—	—	12	—	—	—
Type 3 (sex not determined)	—	32	—	—	23	—	—	21	—	—	—	—	3
*Type 4 (sex not determined)	—	2	—	—	—	—	—	2	—	—	—	—	—
Turkey Point, Florida													
*Type 1, clone #1 (sterile)	—	21	—	—	—	21	—	21	21	—	—	—	—
Type 1, clone #2 (sterile)	—	25	—	—	—	25	—	25	—	—	—	25	—
*Type 2 (sterile)	—	11	—	—	—	11	—	11	11	—	—	—	—

and there is no deviation from Hardy-Weinberg equilibrium (goodness of fit G-test for pooled data, $G = 4.190$, d.f. = 3, $0.5 > P > 0.1$). One individual heterozygous for the B allele and a rare allele product intermediate in electrophoretic mobility to the B and C allozymes was included with the BB individuals in the calculations.

DISCUSSION

Entire local populations of *Haliplanella luciae* commonly disappear suddenly, or individuals may become extremely rare. Numerous examples have been provided by Parker (1919), Allee (1923), Stephenson (1935), and in personal communications from the following persons at the indicated localities: B. L. Bayne (population near the Institute for Marine Environmental Research Laboratory at

TABLE III

Genotypic distributions and allele frequencies of phosphoglucose isomerase in Diadumene leucolena *from Narrow River, Rhode Island, including G-test analysis for goodness of fit to Hardy-Weinberg expectations. Expected numbers are given in parentheses.*

AA	AB	BB	AC	BC	CC	N	$G_{[3]}$
1 (0.16)	5 (6.19)	60 (60.05)	0 (0.50)	11 (9.71)	0 (0.39)	77	4.190, $0.5 > P > 0.1$

$f(A) = 0.046$; $f(B) = 0.883$; $f(C) = 0.071$.

Plymouth, England); D. F. Dunn (population of type 2 individuals in San Francisco Bay, California); C. P. Mangum (populations of type 1 and type 3 individuals at Indian Field Creek, Virginia); and our own observations (population studied by Sassaman and Mangum, 1970, at the Barnstable Town Dock, Massachusetts). As has been noted by Mayr (1963, p. 530) and Dobzhansky (1970, p. 249), such is a common fate of peripheral isolates or founder populations. The latter author comments that such populations represent the species at the limits of its ecological tolerance and may routinely be subjected to conditions beyond the tolerance limits.

The population at Blue Hill Falls, Maine, exemplifies the founder effect in the extreme—all of the anemones in an area of approximately 2000 square meters, in which the population density reaches 4000 individuals/m^2 (Shick, 1976), are members of a single clone. The consequences of a monoclonal population structure become evident upon examination of the results of the survival experiments presented in Shick (1976). Briefly summarizing these, *Haliplanella* shows 100% survival for two weeks at most temperature and salinity combinations from 1.0° C to 27.5° C and 0.5 to 35‰. However, a slight increase in the severity of conditions results in sudden extensive mortality: survival decreases from 95% at 22.5° C and 2.5‰ to 5% at 22.5° C and 1.0‰, and from 90% at 25.0° C and 2.5‰ to 0% at 27.5° C and 2.5‰. Thus, although *Haliplanella* is highly resistant to extremes of temperature and salinity, when the severity of conditions approaches the tolerance limits, there is an abrupt and massive incidence of mortality rather than a gradual elimination of individuals whose genotypes confer less resistance, since only a single genotype is present. Because other populations of this anemone typically are composed of one or a very few clones (Table II), the well known susceptibility of such populations to local extinction is not just understandable but even to be expected.

Factors other than temperature and salinity (*e.g.*, the biological factors of predation, competition, or disease) may also be involved in such local disappearances. We emphasize that the tolerance experiments above employed the criterion of survival for two weeks under stressful conditions, although salinities below 10‰ are ultimately lethal (see Shick, 1976).

The immense success of the BHF population is remarkable in that this location presents an extreme thermal environment (Shick, 1976) and is well beyond Salem, Massachusetts, the species' former known distributional limit on the North American Atlantic coast (Parker, 1919). This success is in large part due to the physiological adaptation to cold in the Maine individuals relative to members of more southern populations, an adaptation probably in existence before the species was introduced locally, in view of the short time involved. The species is not listed in Procter's (1933) survey of the region and was likely introduced in a shipment of oysters (*Crassostrea gigas*) imported from Japan and deposited in April, 1949, in Salt Pond, Sedgwick (Dow, 1970), the tidal outflow of which is Blue Hill Falls. A Japanese origin for this population is circumstantially supported by the much larger size (basal diameters of 1.5 to 2.0 centimeters being common) of these individuals relative to others on the Atlantic coast, and Hand (1955b) has remarked on the larger average size of Japanese specimens. Further, BHF individuals are now known to reproduce asexually by longitudinal fission and by constrictive pedal laceration (Johnson and Shick, 1977), a situation hitherto unreported in populations outside Japan. Torrey and Mery (1904) and Stephenson (1929) deny the existence of more than one means of asexual reproduction in a given population, but it has in fact been recently observed in *H. luciae* from Shôbuta (Atoda, 1976).

Fertilization, and embryonic and larval development in the species remain to be described. *Haliplanella* is known to be dioecious, however, ripe male and female gonads having been seen in different populations; considering the high energetic cost of their production and maintenance (Chia, 1974; Giese and Pearse, 1974), they are presumably functional and adaptive in bisexual populations, which through chance have not yet been observed. The widespread occurrence of individuals having very small body size (see Stephenson, 1935; also, Barnstable Town Dock and Turkey Point, in the present study), typically a reflection of stress, and having sterile mesenteries rendering sex undeterminable (populations listed immediately above), supports the statement of Chia (1976) that asexual reproduction in sea anemones is favored under stressful conditions. Certainly a reproductive mode which obviates the necessity for nutrient-laden, costly-to-produce gonads, is much less expensive energetically.

More direct evidence for the absence of sexual reproduction in the mixed clonal populations at Charlestown Salt Pond and Turkey Point is provided by the observation that not all possible allelic combinations (expected in sexually reproducing populations containing heterozygotes) are present (Table II), unless there is total selection against the missing homozygotes. This does not imply that extreme selection does not occur in isolated *Haliplanella* populations; it may be significant that those in the two most physically rigorous habitats studied (BTD and BHF) are monoclonal, but it is not known whether this is the result of selection among several original clones at both sites or of the fortuitous introduction of single genotypes conferring predaptation to local conditions. The large differences in population size among the four morphological types present at CSP (Table II) may be indicative of the selective elimination of all genotypes but those best suited to that environment. The lack of success of the type 1 and type 4 anemones may not be entirely due to rigors of the physical environment, however, but perhaps also involves interclonal competition and aggression by the well-established type 2 and type 3 anemones that predominate at CSP.

The population at Barnstable Town Dock, which had a density of approximately 1000 individuals per square meter as recently as 1969 (Sassaman and Mangum, 1970), yielded only nine type 3 specimens from an area of 9 square meters during one hour of searching by us in June, 1976. Unfortunately, this population had not previously been characterized as to morphological type or genetic composition. Fixed heterozygosity at the PGI and HK loci (Table II) is good evidence that these nine individuals were members of one clone, which had become more abundant and widespread by August, 1976 (R. J. Hoffmann, University of Pittsburgh, personal communication; and Table II, for sample size). No specimens of *H. luciae* could be found at this site in July, 1977.

Asexual reproduction provides a mechanism for rapid colonization of a new environment through the production of multiple copies of a genotype which has proved to be successful under the local conditions. The continued success of specific populations of *Haliplanella* in the absence or near absence of genetic variation is undoubtedly a consequence of its eurytolerant nature (see Shick, 1976), an example of the "phenotypic plasticity" characteristic of colonizing species (Baker, 1965; Lewontin, 1965).

Baker (1965) has developed the concept of the "general purpose genotype" relative to weedy plants, in which there has been selection for a genotype or series of genotypes conferring broad environmental tolerance. This would allow an introduced species to build up a large population rapidly, without the loss of time involved in the selection of a unique, locally-adapted variant. This concept may also be applicable to populations of *Haliplanella luciae*, which present extreme cases of the founder effect, and which evidently rely on genotypes conferring the potential for successful physiological compensation over a wide range of environmental conditions at a given locality (*e.g.*, Blue Hill Falls). The eurytolerance of other clones reported in the literature, however, is likely not correlated with a single particular genotype, in view of the genetic diversity among clones reported in Table II.

Baker (1972) has observed that plant weeds, in which reliance on general purpose genotypes is common, are especially successful in habitats vacant of pests and competitors, and Selander and Hudson (1976) have suggested that the strategy of general purpose genotypes may fail where a colonizing species regularly co-occurs with genetically variable, rapidly evolving competitors, predators, and parasites. The highly successful BHF clone of *Haliplanella*, employing a suite of adaptations to extremes of temperature, salinity, and desiccation, monopolizes a habitat unoccupied by other locally occurring sea anemones. Both the epifaunal *Metridium senile* and the infaunal *Ceriantheopsis americanus* are present at this site, but exist subtidally or in the lowest reaches of the littoral where they are exposed only by spring low tides. Whereas subtidal populations of *Haliplanella* do occur (viz., CSP), the species at BHF is strictly intertidal, and the intertidal zone may represent a "refuge" for the anemone. Although there is no evidence of aggression by either *Metridium* or *Ceriantheopsis*, *Haliplanella* is heavily preyed on by the nudibranch *Aeolidia papillosa*, which is extremely abundant subtidally. This situation is reminiscent of that in *Mytilus californianus* on the Pacific Coast, where the mussel is restricted to the intertidal by subtidal populations of the predatory asteroid *Pisaster ochraceus* (Paine, 1974). An increased frequency of asexual reproduction under subtidal or low intertidal conditions (Johnson and Shick, 1977) may compensate for the heavy toll taken by the nudibranch and maintain a high population density down to mean low water.

In addition to those of producing multiple copies of successful genotypes and of offsetting losses due to predation, asexual reproduction also provides more immediate advantages manifested through living in dense aggregations (discussed for *Anthopleura elegantissima* by Francis, 1976): first, reduction of effective surface area, resulting in both diminished desiccation during intertidal exposure, and in reduced drag in wave-swept habitats; secondly, aggressive physical exclusion of interspecific and nonclonemate conspecific competitors; and thirdly, cooperation in food capture. Some of these may be important in *Haliplanella*, others less so.

Regarding the first point, increased longitudinal fission and a resultant aggregated spatial arrangement does not appear to occur preferentially under desiccating conditions in *Haliplanella*, since Uchida (1936) observed large, regularly-striped individuals (indicative of minimal fission) in the *high* intertidal, and small, irregularly-striped (regenerating after fission) specimens *lower* in the intertidal. The maintenance of large individual body size would also serve to minimize surface area for evaporation (Ottaway, 1973). Laboratory experiments (Johnson and Shick, 1977) have shown that fission is less frequent, and individual body size and percentage water content larger, in *Haliplanella* experiencing variable, as opposed to continuous immersion, when feeding is equal. In the BHF population, desiccation resistance is also a result of microhabitat features, since the anemone occurs under rocks and among sand, gravel, and shell debris. Finally, the aggregation-related advantage of reduced drag does not appear to be important, since *Haliplanella* typically does not occur in unprotected wave-washed areas on the open coast (Stephenson, 1935; Hand, 1955b; Shick, unpublished observations).

Apropos of the second point, the only record of intraspecific aggression in *H. luciae* is for Japanese specimens reported in a review by R. B. Williams (1975), who discusses the aggressive function of catch tentacles. If the presence of catch tentacles is in fact indicative of aggression against nonclonemates (analogous to the function of acrorhagi in *A. elegantissima* described by Francis, 1973b; 1976), then the extreme rarity of their occurrance in *H. luciae* (Carlgren, 1929; Weill, 1934; both cited by R. B. Williams, 1975) indirectly supports the postulated generality of a monoclonal population structure in isolated colonies of this species. Indeed, the only population of *H. luciae* in which we have observed catch tentacles is the one at CSP, where all four of Uchida's morphological types are present, and the individuals in question were attached to an oyster clump containing both type 2 and type 3 specimens. *Haliplanella* has not been observed to use catch tentacles in interspecific aggression, however, and *Haliplanella, Diadumene,* and *Metridium* often coexist on the same rock.

Regarding cooperation in food capture, the third point raised by Francis, Hausman (1919) observed that single specimens of *Haliplanella* often failed to capture large beach fleas (*Orchestia*), but that the success rate was greater in aggregations of anemones where the tentacles of several contiguous anemones were brought to bear.

Selander and Hudson (1976), referring to work with plants and animals, emphasize that the understanding of the adaptive significance of different breeding systems must include consideration of a multitude of genetic and ecological factors. From the foregoing it is apparent that an understanding of asexual reproduction in sea anemones is enhanced by a detailed knowledge of the biology of the species in question. In a given population of *Haliplanella luciae,* the frequency and even the particular mode of asexual reproduction may be determined by an interacting network of physical and biological factors including temperature, exposure to desiccation, nutritional conditions, and pressure from competitors and predators. The anemone has both the physiological hardiness and the asexual potential for rapid colonization of extreme environments (*e.g.,* the Blue Hill Falls intertidal) uninhabitable by otherwise sympatric potential competitors and predators.

A comparison of *Haliplanella luciae* and *Diadumene leucolena* sheds further light on the evolution and consequences of sexual and asexual reproduction in sea anemones. The two species are sympatric over much of their range on the Atlantic coast of the United States, frequently occurring on the same rock. Both species are notably euryhaline actinians and are similarly abundant in estuaries, and make a quantitatively identical use of free amino acids for cellular volume regulation (Pierce and Minasian, 1974; Shick, 1976). Populations of both species in Virginia are warm-adapted and show the comparatively rare pattern of inverse thermal acclimation of metabolic rate (Sassaman and Mangum, 1970).

Despite their similarities in Atlantic coast distribution and physiological responses to physical environmental factors, they are very different in their reproductive biology. Hand (1955b), based on the regularity of arrangement of the mesenteries, concludes that asexual reproduction does not occur in *Diadumene leucolena,* and our experience with living specimens supports this view. The conformity of genotypic frequencies in the Narrow River population to Hardy-Weinberg expectations (Table III) indicates that sexual reproduction is occurring there.

Evaluation of the proposed advantages of asexual reproduction may provide a clue concerning its absence in *D. leucolena.* The species is indigenous to the Middle Atlantic coast of the United States (Hand, 1955b), being especially common in sheltered estuarine habitats. It does not occur in wave-washed areas, and avoids desiccation in its typical microhabitats of empty barnacle tests and crevices among oyster clumps; thus, the twofold pressure to form asexual aggregations for surface area considerations is minimal. More important perhaps is the reduced competition with other native sea anemones for available hard substrate (an important consideration in epifaunal forms: Francis, 1976; Sebens, 1976), since the anemone fauna of the U. S. mid-Atlantic littoral is comparatively impoverished. This hypothesis is supported by the reproductive situation in other members of the genus *Diadumene* native to the U. S. Pacific coast, which has a diverse anemone fauna (Hand, 1955a, b): *D. franciscana* and *D. lighti* reproduce both sexually and asexually. Similarly, the British species *D. cincta* reproduces asexually, and the well-known diversity of the British sea anemones has been treated in the remarkable volumes of Gosse (1860) and Stephenson (1929, 1935).

Unlike monoclonal *H. luciae,* which shows a sudden and near total incidence of mortality as the tolerance limits for temperature and salinity are approached (Shick, 1976), the sexual maintenance of genetic variability in *D. leucolena* is reflected in a more gradual increase in mortality with increasing stress (Shick, unpublished). This is consistent with the proposal that sexual reproduction and genetic variability ensure population survival and promote evolution by reducing the likelihood of extinction (see G. C. Williams, 1975).

In August, 1976, the synchronous epidemic spawning of male and female *D. leucolena* occurred in a laboratory holding tank, and the previously unobserved embryonic and early larval development was followed. The light brown eggs (mean diameter 98 μm) adhered to the substrate at the bases of the spawning females, where they were fertilized by sperm released by the males. Fertilization success is no doubt enhanced by the eggs remaining *in situ,* rather than being broadcast into the water column. Early cleavage was symmetrical, and the blastula stage was achieved by 8 hours, and the planula larva stage by 12 hours, after fertilization at 17.5° C and 25‰. The planulae remained planktonic for at least five days.

Planktonic dispersal is especially important in an environment that is patchy with respect to available hard substrate. Epifaunal animals in the Chespeake Bay system (where *D. leucolena* is particularly abundant) are most prolific on subtidal oyster reefs, which are often separated by large expanses of soft bottom unsuitable for attachment by adults or settlement by metamorphosing larvae. Planktonic dispersal may thus assume the role of a means of colonizing new habitats, as well as maintaining good local recruitment (see Cory, 1967, and Sassaman and Mangum, 1970, for population size data). The greater success of *Haliplanella* as a colonizer (indicated by its occupation of a much wider geographic range and a greater variety of local habitats) is due not only to its ability to build up large populations quickly *via* asexual reproduction (*e.g.,* the resurgence of the species at BTD between June and August, 1976), but also to its eurytopic nature. While *Diadumene* is more restricted to hard, stable substrates, *Haliplanella* not only occupies rocks and oyster reefs, but also attaches to shell debris, gravel, and marine macrophytes such as *Ulva, Fucus, Zostera,* and *Ruppia.*

A limitation of sexually produced planktonic larvae as a colonizing mechanism [other than the obvious requisite presence of fertile individuals of both sexes, or of a fertile hermaphrodite (Dunn, 1975)] is that the range of environmental conditions permitting sexual reproduction is typically much narrower than that permitting mere survival. Thus, although *Diadumene leucolena* might survive introduction into a new environment, the ecological requirements for gametogenesis and spawning will not necessarily be met. If spawning should occur in an extreme environment, the intense selection predicted by G. C. Williams' (1975) models on animals with planktonically dispersed larvae would be even greater, presumably resulting in low local recruitment. Further, this strictly sexual anemone would have no prospect for the rapid intact preservation of the relatively few successful genetic combinations adapted to the immediate environmental conditions.

Intermediate to the two reproductive extremes presented by *D. leucolena* and populations of *H. luciae* studied to date is the condition exemplified by *Anthopleura elegantissima* and *Metridium senile.* The latter two species employ both sexual and asexual means of reproduction (for discussions and references, see Chia, 1976; Francis, 1976; Hoffmann, 1976). The availability of these alternatives allows for the ameiotic preservation of a proven successful genotype without sacrificing the genotypic plasticity provided by recombination through outcrossing. This situation is also likely the case in bisexual populations of *H. luciae,* which fortuitously have not yet been observed. Taken in their ecogenetic and evolutionary context, the present study and those of Francis (1976) and Hoffman (1976) support the reasonable conclusion that the optimum evolutionary potential involves a combination of sexual and asexual reproduction.

We are grateful to the numerous individuals cited in the text who have contributed to our understanding of asexuality in sea anemones through their provision of information and specimens. Larry G. Harris kindly confirmed the identification of *Aeolidia papillosa.* We thank Cadet Hand, Richard J. Hoffmann, Richard K. Koehn, and George C. Williams for their suggestions concerning the manuscript. This research was supported in part by a grant from the University of Maine Faculty Research Fund.

SUMMARY

Populations of *Haliplanella luciae* on the Atlantic coast of North America typically are composed of one or a few strictly asexual clones. The lack of genetic variability in one local population, that at Blue Hill Falls, Maine, is reflected in the population's response to extremes of temperature and salinity. As the limits of tolerance are approached, there is an abrupt and epidemic incidence of mortality, rather than a gradual one. Genetic and the concomitant physiological uniformity explain the well-known tendency for local populations of *Haliplanella* to disappear suddenly and illustrate a common outcome of the founder effect.

The success of *Haliplanella* as a colonizer is due to its extreme hardiness toward physical environmental factors and to its prolific asexual nature. Asexual reproduction by longitudinal fission and by pedal laceration not only provides a means of rapid colonization of a new habitat, but also a means of producing multiple copies of genotypes that have proved to be successful under local conditions. The resultant population structure recalls that in other animal and plant colonizers, in which there is heavy reliance on general purpose genotypes in isolated populations.

The comparative lack of colonizing success in the ecologically similar anemone, *Diadumene leucolena*, relates to its reproductive biology. Not only is the asexual potential for the rapid spread of successful genotypes lacking, but sexual reproduction typically requires a more optimal set of environmental circumstances which will not necessarily be met in a new habitat.

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TEMPERATURE DEPENDENT CILIARY RHYTHMICITY IN *MYTILUS EDULIS* AND THE EFFECTS OF MONOAMINERGIC AGENTS ON ITS MANIFESTATION

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Some ciliated cells are directly influenced by temperature changes. In *Mytilus edulis* the same relationship can be demonstrated for frontal cilia of isolated gill filaments (Gray, 1929; Hirasaka, Hoshi and Nagumo, 1957; Hoshi and Hoshiyama, 1963). Lateral cilia of isolated gill of *M. edulis* exhibit this temperature effect only if they are first stimulated to beat by the addition of exogenous agents (Aiello, 1960). The need for artificial stimulation to induce lateral ciliary activity of isolated, denervated gill suggests the presence of endogenous innervation mechanisms.

Lateral ciliary activity of *M. edulis* is known to be peripherally dependent on serotonin and dopamine (Aiello, 1962, 1970; Gosselin, 1961; Gosselin, Moore and Milton, 1962; Aiello and Guideri, 1964, 1965, 1966; Paparo and Aiello, 1970). Electrical stimulations of the branchial nerve can either excite or depress lateral ciliary activity (Aiello and Guideri, 1966; Paparo and Aiello, 1970). Recent morphological pharmacology studies (Stefano and Aiello, 1975; Stefano, Catapane and Aiello, 1976) and investigations into the central nervous system (CNS) regulation of ciliary activity (Catapane, Aiello and Stefano, 1974; Catapane, 1976; Catapane, Stefano and Aiello, 1976) have shown that the CNS is composed in part of serotonergic and dopaminergic neurons. The majority of the serotonin content of gill is dependent upon axonal transport of tryptophan hydroxylase from the CNS *via* the branchial nerve. Environmental temperature changes produce changes in endogenous serotonin levels which appear to change first in the CNS (Stefano and Catapane, 1977a). The evidence suggests a dual antagonistic innervation of the lateral ciliated cells originating from the cerebral and visceral ganglia.

The present study sought to investigate the nervous control of lateral ciliary activity in nature as well as in the laboratory. The possibility of rhythmic activity was examined, along with several environmental factors which may influence it.

MATERIALS AND METHODS

Field study

Subtidal specimens of *M. edulis* were observed *in locus* at Island Beach State Park, New Jersey, by means of scuba diving. During dark hours a portable underwater light was used. Slightly open animals were selected for use. Their posterior adductor muscle was severed while underwater, and the animals were dislodged from their clusters. Jørgensen (1975) has shown that cutting the posterior adductor muscle does not impair the health of the animals nor effect the rate of water transport by the gill. At the time of collection, the water temperature at the level from which the animals were harvested was recorded and a quantity of this water was taken in which to transport them. The delay between the time of collection to the time of observing ciliary activity was approximately 30 min, during which time the animals remained at a constant temperature in five gallons of sea water gathered from their level. Animals were collected and ciliary rates observed hourly for 24 hour periods on June 10, 21 and August 23 and at irregular intervals during the months of June, July and August.

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When pharmacological pretreatments were part of the experiments, animals were injected into the posterior adductor muscle while underwater. The drug solution was prepared and carried in labelled syringes prior to diving. Each drug was administered in a 0.1 ml volume of filtered sea water. Color coded nylon threads were used to identify animals and drug treatments. At the appropriate times, animals were transported quickly to the nearby observation site, where lateral ciliary activity was observed stroboscopically according to the method of Gray (1930) and Dral (1967).

Laboratory study

Specimens of *M. edulis* were collected at Long Island Sound, New York, at New Rochelle, transported to the laboratory and maintained in artificial sea water (ASW) prepared from Instant Ocean Sea Salts, at a pH of 7.0–7.4, specific gravity of 1.022–1.026, with a constant aeration supply and varying temperature and time periods depending upon the experiments.

Whole animal preparations consisted of deshelled animals positioned in petri dishes. The mantle and the lateral gill lamina from the right side of each animal were excised to allow the cilia on the medial lamina to be viewed with transmitted stroboscopic light. The cerebral and pedal ganglia remained connected to the visceral ganglia and the gill; however, the visceral commissure was transected. These whole animal preparations maintained all neuronal connections except those linking the two visceral ganglia. The beating rate of each preparation was measured each minute for ten minute periods. The mean ± s.e.m. of the values were calculated from all the preparations observed for each type of experiment. During the time course of the observation periods, the temperature of the medium bathing the animals did not vary by more than 2° C.

Visceral ganglion (VG) preparations were composed of only the gill and the visceral ganglion connected by the branchial nerve. Electrical stimulations were delivered by platinum needle electrodes with a Harvard 342 Stimulator. Repeating bipolar pulses at 0.1 Volts and 5 or 50 Hz for three minutes were applied to the branchial nerve.

OBSERVATIONS AND RESULTS

Field study

Specimens of *M. edulis* observed underwater had their valves slightly open for the majority of the time they were viewed. They would close their valves in

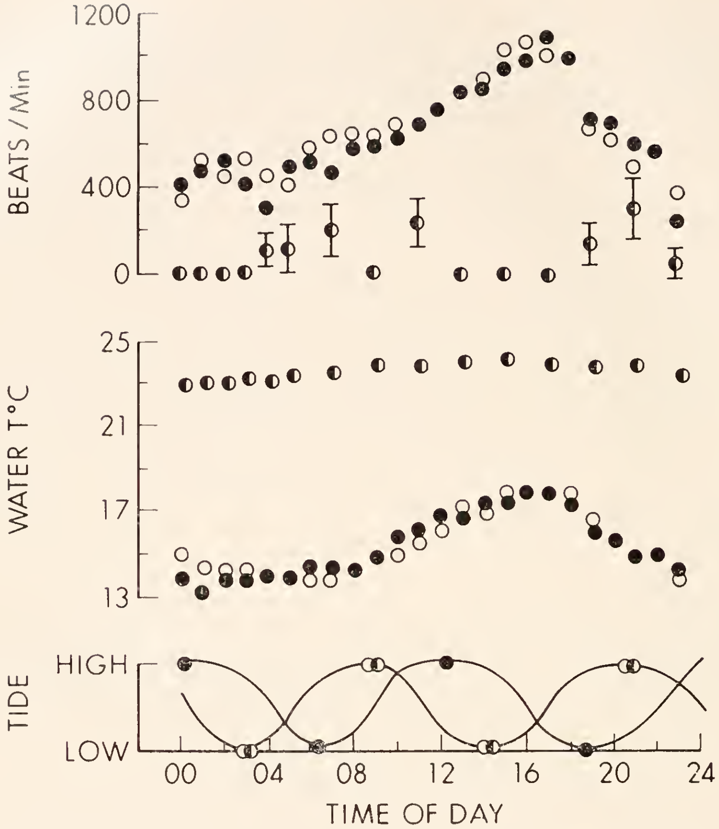

Figure 1. Graph showing the relationships between tides, water temperature (°C) and beating rates (beats/min) of lateral cilia of whole animal preparations of *M. edulis*. Open circles represent June 10, solid circles June 21, and half solid circles August 23. The tidal cycles for June 10 and August 23 are almost identical, so only one line is shown. The beating rates for each hour represents the mean of the average of 10 one minute interval readings/animal for five to ten animals. In all cases except where a bar shows s.e.m., the s.e.m. was less than 10% of the beating rate.

response to a disturbance and reopen them at a later time. Those animals which were collected hourly for 24 hour periods and had their lateral ciliary activity assessed showed a diurnal ciliary rhythm (Fig. 1). For June 10 and 21 the activity appeared to be independent of tidal cycles, but rose and fell in coordination with the daily rise and fall of the water temperature and the sun. For August 23 no ciliary rhythmicity was observed, and the water temperature remained fairly constant. To examine environmental influences more closely, groups of animals were collected between June 6 and July 27, on 16 different occasions at different tidal periods, and their beating rates were plotted against the tidal period and temperature at which they were collected (Fig. 2). Examination of these figures reveals that a linear regression with a correlation coefficient of 0.8 can be plotted for the plot against temperature, but the plot against tidal period does not show a corresponding relationship.

In order to determine the influence of the nervous system on the rhythmicity of ciliary activity, animals were injected with neurotransmitters and drugs which affect neurotransmitter metabolism. Animals for similar experiments were uniformly treated, collected and observed at the same time so that the behavioral modifications were not due to different groups being collected at different periods of their temperature-activity cycles.

The lateral cilia of whole animal preparations were observed after quickly transporting them from their ocean environment to the field observation station. Prior to their collection, the posterior adductor muscle of slightly gapping animals was injected *in locus* with 200 μg of serotonin, dopamine or acetylcholine or 100 μg of 6-hydroxydopamine (6-OHDA), alpha methyl-para-tyrosine (AMPT) or 5,6-dihydroxytryptamine (5,6,-DHT) dissolved in 0.1 ml of filtered sea water for varying time periods. Controls were vehicle injected. No mortalities were observed for at least two weeks as a result of these injections.

Table I shows that the average basal activity of the control group was 562 beats/min. Those animals which were pretreated two hours earlier with serotonin displayed substantially higher basal beating rates. The mean rate was 1039 beats/min, about twice the value of untreated animals. Animals injected with dopamine for the same two hour interval were not beating at all. The animals injected with acetylcholine did not differ in their activity from that of the controls.

Another set of animals were observed four and six hours after injections. They displayed similar activity as did the two hour groups. Table I shows the basal beating rates of sets of animals observed 17 and 21 hours after injections. The control groups were beating at 740 for the 17 hour reading and 848 beats/min for

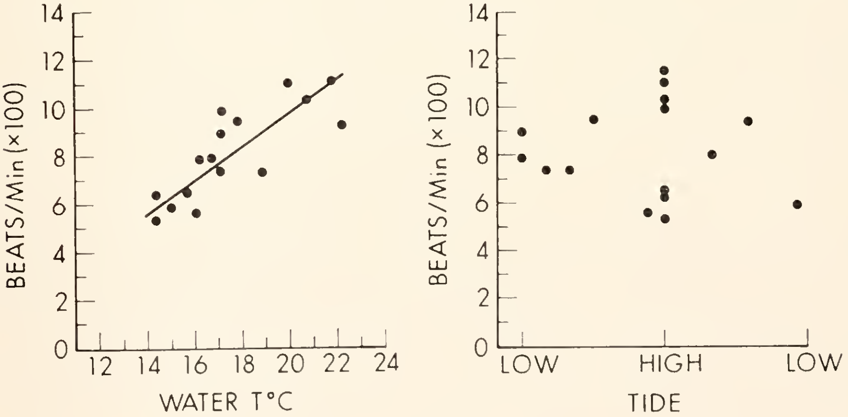

FIGURE 2. Lefthand graph shows the relationship between the temperature at which the animals were collected and lateral ciliary beating rates (beats/min) of whole animal preparations. Each point represents the mean values of five animals. The s.e.m. for each point is less than 10% of the beating rates. The best fit line is drawn on the graph. The correlation coefficient r is 0.82. In the righthand graph, the same beating rates are plotted against the cycle of the tide at which the animals were collected. No correlation exists.

TABLE I

Lateral ciliary activity (beats/min ± s.e.m.) of whole animal preparations. Groups of animals were injected while underwater with the indicated drugs and their cilia observed at the specified time intervals; 200 µg of serotonin (HT), dopamine (DA) or acetylcholine (ACH) were injected into the posterior adductor muscle in a 0.1 ml volume. Control animals were injected with 0.1 ml of sea water. N is the number of animals for each treatment.

Treatment	Time after injection (hr)	N	Ciliary rate (beats/min ± s.e.m.)
SW	2	5	562 ± 43
HT	2	5	1039 ± 24
DA	2	5	0
ACH	2	5	608 ± 16
SW	6	5	943 ± 33
HT	6	5	1173 ± 25
DA	6	5	0
ACH	6	5	932 ± 33
SW	17	5	740 ± 23
HT	17	5	1024 ± 56
DA	17	5	0
ACH	17	5	821 ± 40
SW	21	5	848 ± 16
HT	21	5	1194 ± 73
DA	21	5	620 ± 73
ACH	21	5	790 ± 29

the 21 hour reading. Those animals which were injected earlier with serotonin maintained high basal rates. At 17 hours it was 1024, and at 21 hours it was 1194 beats/min. The dopamine-injected animals had cilia which were still quiescent after 17 hours. For the group injected group injected 21 hours earlier with dopamine, the cilia were beating at 620 beats/min. Animals injected with acetylcholine did not display any altered ciliary activity at any of the time intervals at which they were observed.

Animals were administered 6-OHDA and AMPT which specifically interfere with dopaminergic mechanisms, and 5,6-DHT which interferes with serotonergic systems in an attempt to interfere with monoaminergic mechanisms. Because these agents exert neurotoxic and metabolic effects, longer time periods were necessary to observe the behavioral effects. The whole animal preparations showed altered ciliary activity in response to each drug treatment (Table II). Basal activity of animals observed four days after injection with 6-OHDA was 1002 beats/min as compared to 603 for the vehicle injected controls injected at the same time. The basal rate of animals injected with AMPT was 904 beats/min as compared to controls which were beating at 475. The set of animals which were injected with 5,6-DHT three days earlier were not beating when observed. The controls for this experiment were beating at 576 beats/min.

Electrical stimulation to the branchial nerve can elicit cilio-excitation or inhibition depending on the stimulus parameters employed (Aiello and Guideri, 1966; Paparo and Aiello, 1970). The cilio-excitatory response is dependent on peripheral release of serotonin, while the inhibitory response is due to dopamine. Therefore, the effects of branchial nerve stimulation in animals pretreated with

6-OHDA, AMPT or 5,6-DHT were studied. VG preparations were chosen for these studies so that incoming nervous activity from the cerebral ganglia would not interfere with and obscure the interpretation of the results and so that the preparations conformed to the type used by the earlier works which established the stimulus parameter relationships. For each preparation the branchial nerve was stimulated with a 5 or 50 Hz bipolar 2 msec duration pulse and 0.1 Volts for 3 min, after observing basal rates.

While whole animal preparations tend to display spontaneous activity, a higher number of VG preparations tend to be inactive. Figure 3 shows that for control animals, stimulations at 5 Hz substantially accelerated beating, while stimulations at 50 Hz decreased ciliary rates. The response to 50 Hz stimulation is not always seen for all the gill filaments in the field of view. However, those filaments that do respond, only decrease their beating rates after a few second latency period, and repeatedly do so in response to repeated stimulations at later times.

Animals which were pre-injected four days earlier with 6-OHDA displayed an altered response to stimulations (Fig. 3). When 5 Hz was employed, they increased their beating rates from 0 to 800 beats/min within 3 min. When 50 Hz was employed, they only decreased their rates slightly to 630 beats/min as compared to 125 beats/min for the controls. The cilia of the control animals did not return to their basal rates during the observation period, while the cilia of the 6-OHDA treated animals did.

Animals which were treated four days earlier with AMPT were similarly tested. The cilia of the AMPT-treated animals responded almost identically as did the cilia of the 6-OHDA-treated animals (Fig. 3).

Animals which were injected three days earlier with 5,6-DHT had no basal ciliary activity. Stimulation at 5 Hz failed to activate the lateral cilia. However, the addition of 10^{-4} M serotonin to the gill medium activated cilia and increased beating rates to 1000 beats/min. Groups of animals were injected with 5,6-DHT and observed at varying time intervals between 12 hours and three days to find a period when the cilia were spontaneously active, to determine if 50 Hz stimulations would be inhibitory. Only the animals which were observed 12 hours after injections had beating rates above 0. For these animals, stimulations at 5 Hz

TABLE II

Lateral ciliary activity (beats/min ± s.e.m.) of whole animal preparations which were pretreated in locus *with 6-OHDA, 5,6-DHT or AMPT (100 µg in a 0.1 ml volume) for the indicated time periods prior to observation. Control animals were injected with sea water. N is the number of animals per group. Statistical significance was determined by a one tailed Student's* t*-test.*

Treatment	Time after injection	N	Ciliary rate (beats/min ± s.e.m.)
6-OHDA	4 days	5	1002 ± 44*
SW	4 days	5	603 ± 38
5,6-DHT	3 days	5	0*
SW	3 days	5	576 ± 56
AMPT	4 days	5	904 ± 53*
SW	4 days	5	475 ± 93

*$P < 0.001$

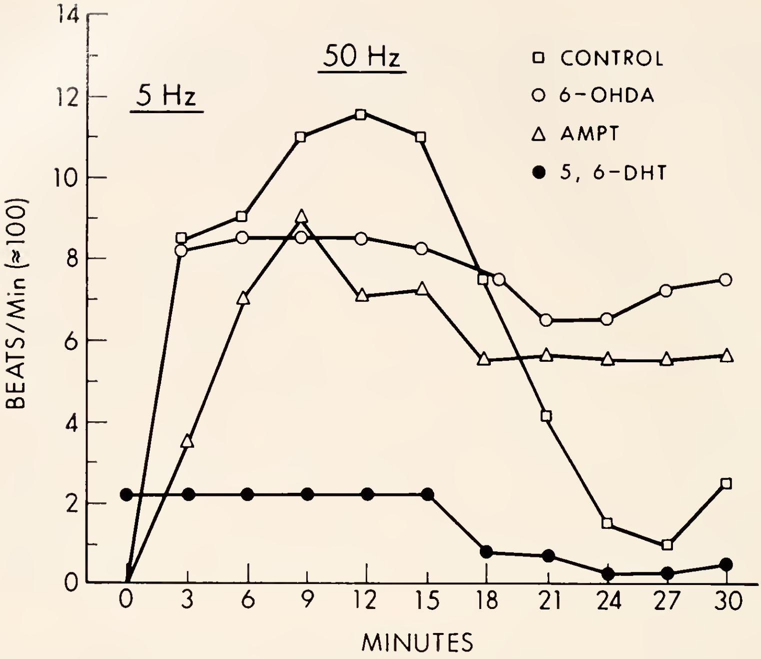

FIGURE 3. Lateral ciliary activity (beats/min ± s.e.m.) of VG preparations is shown. Each point represents the mean of five preparations. The preparations were observed for 15 min before starting the experiments. Groups of animals were preinjected 12 hr earlier with 5,6-DHT or four days earlier with 6-OHDA or AMPT. Electrical stimulation at 5 or 50 Hz was delivered to the branchial nerve at the indicated times. The s.e.m. of each point was less than 10% of the beating rates.

still failed to accelerate beating but stimulations at 50 Hz depressed the cilia (Fig. 3).

Laboratory study

Animals were collected from Long Island Sound during October and maintained at 5–6° C, 15–16° C and 20–24° C in ASW under otherwise identical conditions for six days. Table III shows that lateral ciliary rates are dependent on the temperature at which the animals were kept. All the preparations were observed at the same temperature, 20° C. Groups of animals that were kept at these temperatures but under constant darkness, except for irregularly spaced intervals when the water was changed, were also observed. Lateral ciliary beating did not vary in respect to different lighting cycles during the time course of the experiments.

DISCUSSION

The field experiments demonstrated that ciliary activity of whole animal preparations was not constant but exhibited a diurnal rhythm which was independent of the tides. The effects of temperature changes could not be distinguished from those of light, since they both increased and decreased in unison during the study. The effects of different lighting cycles on this diurnal activity was examined in the laboratory, and it was found that depriving animals of light did not significantly alter ciliary activity. Therefore, the ciliary rhythm observed in nature corresponds with the changing environmental temperature and not with light.

The dependency of lateral ciliary activity on the peripheral transmitters, serotonin and dopamine, has been illustrated in numerous studies. Recent studies involved with the CNS regulation of ciliary beating has shown that both the cerebral and visceral ganglia exert influences on ciliary activity which can be elicited by the application of serotonin and dopamine to the ganglia (Catapane, Aiello and Stefano, 1974; Catapane, 1976; Catapane, Stefano and Aiello, 1976). Applications of serotonin and dopamine to the CNS increased or decreased ciliary rates, respectively. The animals which were injected with serotonin continually beat at higher rates than did controls for up to 21 hours. The animals injected with dopamine showed depressed activity throughout this time period, while the acetylcholine-injected groups showed no variance from controls.

Previously it has been shown by histofluorescence that injecting serotonin and dopamine into the posterior adductor muscle results in their selective accumulation into the nervous system (Stefano and Aiello, 1975). This increase was able to be visualized for at least two days after treatments. The prolonged presence of serotonin and dopamine in the animals had a physiological effect on ciliary activity. The origin of this neurological effect could be a combination of both ganglionic and peripheral actions.

Pharmacologically altering the animal's monoamine content resulted in modifications of the diurnal ciliary activity. Selectively depleting with AMPT (Underfriend, Zaltzonan-Nirenberg and Nagatsu, 1965) or destroying the dopaminergic system with 6-OHDA (Thoenen and Tranzer, 1968) caused an increase in the endogenous basal activity and, correspondingly, an impairment or complete disfunctioning of the inhibitory mechanism. Serotonergic destruction with 5,6-DHT (Baumgarten, Bjorklund, Lachenmayer, Nobin and Steneir, 1971) likewise changed the endogenous basal ciliary activity. Consistently, cilia of animals treated in this manner were quiescent or beating at low rates. Stimulating the branchial nerve did not produce cilio-excitation. However, addition of serotonin to the gill did increase beating rates, showing that the ciliated cell receptor itself was unaltered.

TABLE III

Lateral ciliary activity (beats/min ± s.e.m.) of whole animal preparations. Groups of animals were maintained for six days at the indicated temperature, either in a normal light-dark cycle or in the absence of light. N is the number of animals in each group. Statistically, there is no significance between the groups kept in the dark as compared to those with a light-dark cycle.

	Ciliary rates (beats/min ± s.e.m.)			
	N	Normal light-dark	N	Absence of light
5–6° C	5	0	5	0
15–16° C	5	978 ± 43	5	960 ± 78
20–24° C	5	1178 ± 12	5	1098 ± 35

Jørgensen (1975) concluded that serotonergic innervation plays no important role in regulating the frequency of lateral ciliary beating, but may only be concerned with maintaining lateral ciliary activity. He bases his conclusions on the fact that "the sensitivity (to serotonin) of both lateral cilia and laterofrontal cirri increased with age of the excised gill fragments until advanced stages of tissue disintegration" (Jørgensen, 1975, p. 224), and that serotonin did not change the rate of yeast clearance by undisturbed animals. The sensitivity phenomenon which he describes is exactly what would be expected as a result of supersensitivity from denervation produced by excising gill fragments. The relationship among lateral ciliary beating rates, rate of water pumping and rate of particle filtration has not been adequately described at this time, but is known to vary under differing conditions (Dral, 1967; Hildreth, 1976). Based upon these facts, the well known relationship between the rate of lateral ciliary beating and the dose of serotonin added to gill (Gosselin, 1961; Aiello and Guideri, 1966), and recent studies showing that the rate of lateral ciliary beating increases with increasing concentrations of serotonin applied directly to the visceral ganglion of preparations in which the media bathing the gill is maintained separately from that bathing the visceral ganglion (Catapane, Stefano and Aiello, in preparation), it appears that frequency of lateral ciliary beating may well be dependent upon the serotonergic innervation.

In *Anodonta cygnea,* the catecholamines and serotonin serve not only as peripheral neuroeffectors of the adductors but also in influencing the periodicity of the animals' activity (Salanki and Hiripi, 1970; Hiripi, 1973; Salanki, Hiripi and Nemcsok, 1974a,b). This activity was subject to changes by the use of various drugs (Hiripi, 1973).

Histofluorescent and biochemical evidence supports the proposed drug actions and physiological findings in our experiments. As shown previously, AMPT and 6-OHDA not only lowered dopamine content of the CNS but also produced a detectable rise in serotonin (Stefano, Catapane and Aiello, 1976). The sustained high beating rates and the inability to neuronally inhibit them must be viewed as a combination of a dopaminergic impairment and an increased serotonin content.

Several earlier studies examined the relationship between ciliary activity and temperature in *M. edulis.* These studies were concerned with frontal cilia and measured either particle movements (Gray, 1929, 1930), or gill crawling rates (Hirasaka *et al.,* 1957; Hoshi and Hoshiyama, 1963) of isolated gills. Aiello (1960) studied the temperature-activity relationship of lateral cilia in *M. edulis* and found a Q_{10} of 1.84 for beating rates *vs.* temperature over the range of 2–24° C. In his experiments, however, veratrine sulfate was always added to the gill bathing fluid to initiate and stabilize lateral ciliary activity. In muscular tissue, veratrine acts at the postjunctional and sarcoplasmic membranes to initiate excitatory postjunctional potentials (Goodman and Gilman, 1967, p. 604). Isolated (denervated) gills without this treatment or the addition of potassium tend to have no lateral ciliary activity or very poor and sporadic activity regardless of the temperature (Aiello, 1960; Takahashi and Murakami, 1968). In the present study, the CNS innervation of the gill was left intact. These preparations had stable-active ciliary rates which changed speeds in response to changing temperatures without the need of exogenous stimulations.

Selective destruction of the serotonin system produced by 5,6-DHT accounts for the inability of cilia to beat continuously and for the lack of response to neuronal stimulations. A previous study (Stefano, Catapane and Stefano, 1976) showed the involvement of serotonin in a temperature dependent ciliary rhythm. In the present study, pharmacological alterations of the serotonin and dopamine contents changed this temperature-dependent rhythm. Since isolated gill do not display ciliary rhythmicity with respect to temperature, unless the receptors of the ciliated cells are artificially activated, this indicates that central pathways are involved in the manifestation of this peripheral activity in the intact organism. The interrelationship of these pathways is in need of further investigation in terms of distinguishing central from peripheral mechanisms. Therefore, the natural lateral ciliary rhythm may be regulated in part by the nervous system responding to temperature changes and also may be the result of a direct effect of temperature on the sensitivity of the ciliated cell receptor to nervous stimulation.

In the field during the latter part of August and into September, a greater number of dead mussels were found than during other times of the summer. Cilia of healthy animals were either not beating or were slowly beating with no apparent rhythmicity. The water temperature was warm and in a 24 hour period it did not vary more than 2 degrees. These higher relatively constant environmental temperatures induced an overall higher metabolic rate which could not be nutritionally supported, or the high metabolic rate itself could not be sustained. Therefore, a possible explanation of this irregular activity pattern may be the overall poor health of the animals due to starvation. It has been a general observation in our laboratory that during February a large number of dead and unhealthy animals are seen at collection areas. At this time, the prolonged exposure to low temperature, which lowers CNS monoamine content (Stefano and Catapane, 1977b) and depresses ciliary activity, may also result in starvation.

In conclusion, *M. edulis* contains a temperature-sensitive neuronal mechanism involving a serotonergic system capable of altering ciliary rates. The possible effects of thermal pollution should be considered on the nervous system and overall health of coastal shellfish in view of the fact that constant high temperatures can cause starvation even if a plentiful food supply is available.

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SUMMARY

Lateral ciliary activity of the bivalve mollusc *Mytilus edulis* was studied in the field and in the laboratory. A diurnal rhythm corresponding to the environmental

temperature changes was found. This behavior was modified by treating animals with serotonergic and dopaminergic agents, disrupting the serotonin and dopamine innervation of the cilia. The study shows that manifestation of this temperature-dependent rhythmicity is due peripherally and centrally to monoaminergic pathways.

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EYESTALK REGULATION OF MOLT AND VITELLOGENESIS IN *UCA PUGILATOR*

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Fiddler crabs of the species *Uca pugilator* have the most extensive continuous geographic range of the several species of *Uca* to be found in the United States. From the northern limit of their range, Cape God, Massachusetts, they occur along the east coast to the tip of Florida, along the west coast of Florida, and extend westward on the Gulf coast to Texas (Crane, 1975). Crabs of the genus *Uca* reproduce throughout the year in the tropics, but in temperate regions the breeding season is restricted to a much shorter period. Crane (1975) reported a breeding season of about two months (end of May to early August) for *U. pugilator* on Cape Cod. Salmon and Astaides (1968) reported field observations of courtship activity at Beaufort, North Carolina, where crabs were seen on the beach in early March, but courtship activity was first observed in early April and continued until late August. Herrnkind (1968) stated that approximately 10% of females in burrows were eggbearing in April, and from May to October nearly all females were ovigerous. These latter observations were presumably made near Miami, Florida. Thus, it would appear that northern extension of the range of this species is accompanied by increasing restriction of the breeding season.

Adiyodi and Adiyodi (1970, 1974) have emphasized the generally antagonistic relationship between reproduction and molting. In addition to a regulatory system channeling stored materials either into somatic growth or vitellogenesis, inhibition of molt during egg incubation is required in female decapods. The basic pattern of hormonal regulation of molt has been extensively studied and involves a molt inhibiting hormone (MIH) produced by the X-organ-sinus gland complex and a molting hormone (MH) produced by the Y-organs (Passano, 1960). The hormonal regulation of reproduction is less well understood and the interrelations between molt and reproduction still less so.

Among brachyurans that have been studied, it is not uncommon to find species that spawn more than once in an intermolt. Gomez and Nayar (1965) studied *Paratelphusa hydrodromous*, which has a breeding season restricted to the months March to September. Up to three spawnings occur within a single intermolt, and there is usually a single molt per year. Each vitellogenesis appears to occur in two phases, an initial slow phase, and a terminal rapid one, culminating in oviposition. Adiyodi (=Gomez) (1968) reported that unilateral eyestalk removal does not stimulate ovarian development in December (prior to vitellogenesis) but does stimulate it during January when slow vitellogenesis is occurring. Implants of thoracic ganglia or brain stimulate ovarian maturation during slow vitellogenesis. An ovary stimulating hormone (OSH) is postulated to be produced in the central nervous system.

Ryan (1965) described the pattern for *Portunus sanguinolentus* as nonseasonal both for molt and spawning, but with a relatively fixed interval (about 60 days) between molt and first spawning. Thereafter, the intervals between spawnings, of which there are two more in the same intermolt, are 30 to 40 days each.

Cheung (1969) reported on the molting and spawning pattern for *Menippe mercenaria*. The study involved animals maintained in the laboratory for periods of more than a year as well as freshly collected crabs. Cheung noted that there appeared to be no difference between the two categories with respect to molt or spawning. Spawning was seasonal, peaking in August and September, dropping rapidly in October and remaining very low from November through April. Molts occurred less frequently than spawnings and with less regularity. About half occurred between November and January 31 and thus coincided with low frequency of spawning. However, the other half of the molts were scattered irregularly through the rest of the year. This crab spawns several times in one intermolt with apparently no fixed intervals between molt and first spawning or between subsequent spawnings. Cheung also studied the effect of destalking at various times of year. The results were somewhat ambiguous: molt alone was induced in ovigerous crabs destalked at the end of August and in nonovigers destalked in November; during these same times, molt followed by one or more spawnings was also induced by the operation. Crabs destalked in March when controls molted had the molt accelerated. Spawning alone (*i.e.*, not preceded by molt) occurred in practically all of a group destalked in mid-September, and there were no intact controls for this experiment. Cheung concluded that a number of factors operate to modify the results of destalking, including light, temperature, and salinity.

Bomirski and Klek (1974) described reproduction in *Rhithropanopeus harrisii* in which spawning is restricted to June through August. The reproductive season ends with a molt, following which vitellogenesis proceeds at a slow rate through the winter, reaching completion at first spawning of the next year. In the early part of the breeding season, vitellogenesis accompanies incubation of eggs, and spawning immediately follows hatching. In the latter half of the season no vitellogenesis occurs until after the ecdysis that terminates the season. During the slow vitellogenesis in autumn, destalking stimulates ovarian development; during rapid vitellogenesis in summer and during the resting period in late summer, destalking produces little, if any, stimulation.

Brown and Jones (1949) showed that removal of eyestalks from *Uca pugilator* induced ovarian growth and that implantation of sinus glands prevented such growth in destalked crabs. Their experiments were carried out between July 10 and August 12 at Woods Hole, Massachusetts, and therefore would have been in the latter part of the breeding season, as reported by Crane. Brown and Jones found that only 7 out of 31 controls showed mature ovaries as judged by color, and that no controls laid eggs during the experimental period. They do not mention any molts that may have occurred. Guyselman (1953), also working at Woods Hole, reported destalking females on July 28; all molted between 5 and 21 days after destalking. He noted that molting in the controls was of very low frequency during the study. Abramowitz and Abramowitz (1940) reported destalking *Uca pugilator* during the summer at Woods Hole and thereby stimulating molt. They also stated that the crabs breed in September. However, there is some question about the immediate relevance of this report, since both the photographs and the description suggest that they were using *U. pugnax* rather than *U. pugilator*.

As Bomirski and Klek (1974) have pointed out, because the response of female crabs to eyestalk removal may vary with the species, age, and time of year, experiments on the role of OSH must be coupled with examination of the reproductive cycle. In addition, since the interrelations of molting and reproduction also vary from species to species, the molting cycle must be considered in any study of reproduction.

This study deals with ovarian development and ecdysis in intact crabs and the response to eyestalk removal during the period from September through May for *U. pugilator* from Florida.

MATERIALS AND METHODS

The crabs used in these experiments were obtained from the Gulf Specimen Co., Panacea, Florida. Shipments were received on the following dates: January 13, February 25, April 15, September 15, October 27, and December 31, 1976. The shipments of September, October, and April consisted of freshly collected crabs. The other shipments were of crabs that had been kept in holding tanks from early to mid-November. The holding tanks contained three to four feet of sand in which the crabs made burrows, and the water supply was the local sea water. After arrival at Towson, Maryland, control and experimental crabs were kept in transparent plastic containers with a small amount of artificial sea water (Instant Ocean). Food (pieces of lettuce or spinach) was placed in the containers two to three times a week, at which time the water was changed. During experimental periods crabs were examined daily for ecdysis, and when the first molt occurred, all crabs were isolated and maintained in small finger bowls until they were sacrificed.

Eyestalks were removed by cutting through the flexible membrane at the base, using a scalpel and without cautery. Illumination conditions of three kinds were used: natural illumination (with uncontrolled temperature), constant illumination, and 10L:14D. For the latter two conditions the light intensity was about 40 ft.-c, and temperature was constant at 23° C. Since the effects of photoperiod, if any, were very small compared with the effects of destalking for the processes discussed in this report, the results from all photoperiods are combined. In all periods, at least two of the illumination conditions were studied simultaneously.

The period of observation varied from a maximum of 30 days to 12–15 days, the shorter periods being made necessary when there was a high incidence of mortality. In general mortality was low ($< 10\%$) and occurred within 24 hours of eyestalk removal, in which case death was attributed to the surgical procedure. The experimental design called for examination of ovaries at 10, 20, and 30 days. However, when animals died, they were autopsied, and matching controls were sacrificed. All ovarian weights obtained eight days or more after destalking are included in this report.

Carapace width (CW) and weight of experimental and control crabs were recorded just prior to dissections. The carapace was removed and, on the stage of the dissecting microscope, the ovaries were dissected out and placed in sea water. Any extra-ovarian tissue was removed, and the ovaries were transferred to a previously tared plastic weighing dish. Excess water was blotted up with filter paper and the ovaries were weighed on a Mettler analytical balance. Weights were recorded to the nearest tenth of a milligram. A record was made of the color of the ovaries.

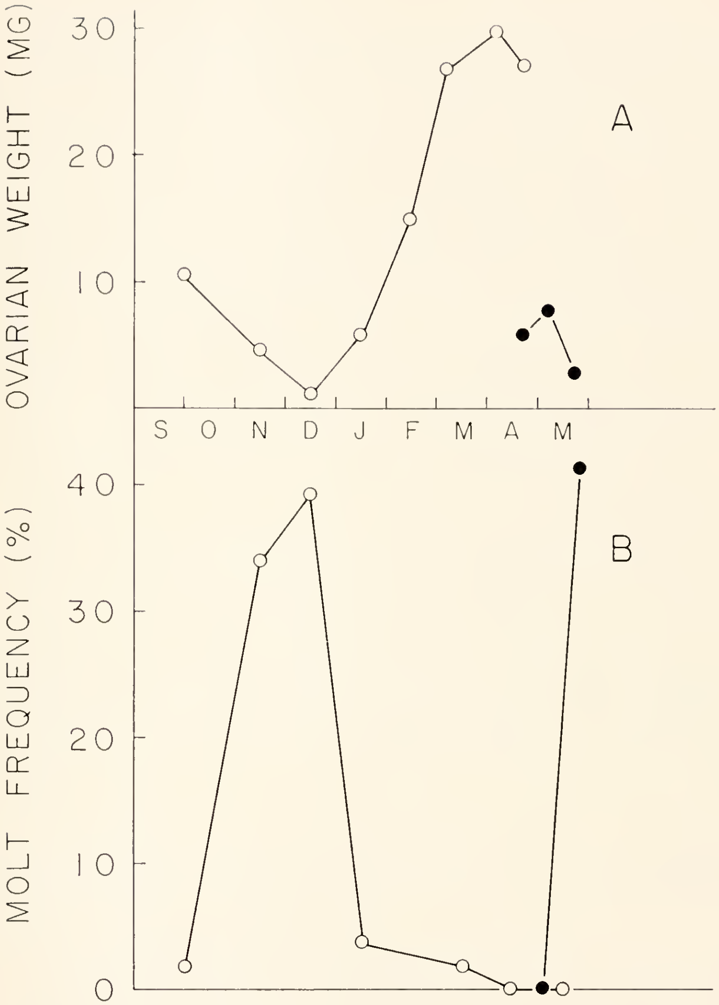

FIGURE 1. Mean ovarian weights (A) and frequency of molt (B) for intact crabs as a function of time of year. Open circles represent nonovigers; solid circles, April ovigers.

For most experiments the animals used were between 14 and 16 mm CW. In a few experiments some crabs were included that were up to 19 mm or as small as 12 mm CW. In no case, however, did the mean CW for any group exceed 16 mm or fall below 14 mm.

RESULTS

Intact crabs

The mean ovarian weights for control crabs in each of the experimental periods are presented in Figure 1A. Although, for reasons that will become obvious, it would not be appropriate to ascribe standard errors of the mean to some points, the curve as a whole indicates a systematic change throughout the year. Ovarian weights decrease from September through December. They begin to increase in January, are well advanced in March, and when egg-laying occurs in a part of the population in April, a considerable decrease in mean weights would be recorded if the crabs were treated as a single population. Approximately 30% of the crabs received April 15 were ovigerous on arrival or laid eggs within the next week. In the rest of the population at that time, 50 to 60% had ovaries that weighed less than 30 mg, thus being below the weights at which egg-laying would be expected to occur. The crabs that were ovigerous in April did not mature another set of eggs during the next month and a half. As will be discussed later, there is evidence that these post-ovigerous crabs undergo molt prior to any further reproductive activity.

Gonadal indices (percentage of wet body weight represented by ovaries) for the experimental periods, together with the variance, number of animals used, and mean CW, are presented in Table I. The gonadal index, like the ovarian weight, reaches a minimum in December and thereafter increases to maximal values in March and April. Mean CW for control crabs ranges from 14.0 mm in December to 15.6 mm in February.

The relative frequency distributions in ovarian weight classes for four groups of crabs are shown in Figure 2A and B. The distributions in Figure 2A are for crabs received February 25, and for nonovigerous crabs received April 15. Both groups represent crabs with near maximum ovarian weights and gonadal indices. There is no single class with as much as 30% of the total number examined, and each of the three to four smallest weight classes contain about the same frequencies. The occupied classes extend up to 130–139.9 mg. Neither of the distributions in Figure 2A represents a "normal" distribution; they obviously come from a highly heterogeneous assemblage to which the application of conventional statistics is not meaningful.

The distributions in Figure 2B, for crabs received January 13 and for the ovigerous crabs received April 15, are quite different. These distributions are characterized by a high frequency in the smallest weight class (0 to 9.9 mg), a rapid drop

Table I

Gonadal indices (percentage of wet body weight represented by ovaries), variance (s^2), number of animals used (N) and mean carapace width (CW) for the experimental periods.

	Destalked				Intact			
	Gonadal index	s^2	N	CW	Gonadal index	s^2	N	CW
September-October	0.94	0.242	33	14.6	0.30	0.015	41	14.6
November	1.17	0.508	22	15.9	0.35	0.036	22	15.0
December	0.481	0.048	14	14.6	0.186	0.022	14	14.0
January	2.85	4.24	31	16.0	0.471	0.10	29	14.6
February	1.62	0.397	13	15.4	0.99	0.518	16	15.6
March	3.22	4.41	51	15.3	2.00	4.202	50	15.3
April (nonovigers)					2.25	1.661	13	15.2
April (ovigers)	1.29	0.58	21	15.6	0.54	0.053	21	15.3
May (post-ovigers)	1.00	0.575	16	15.6	0.438	0.066	16	15.3

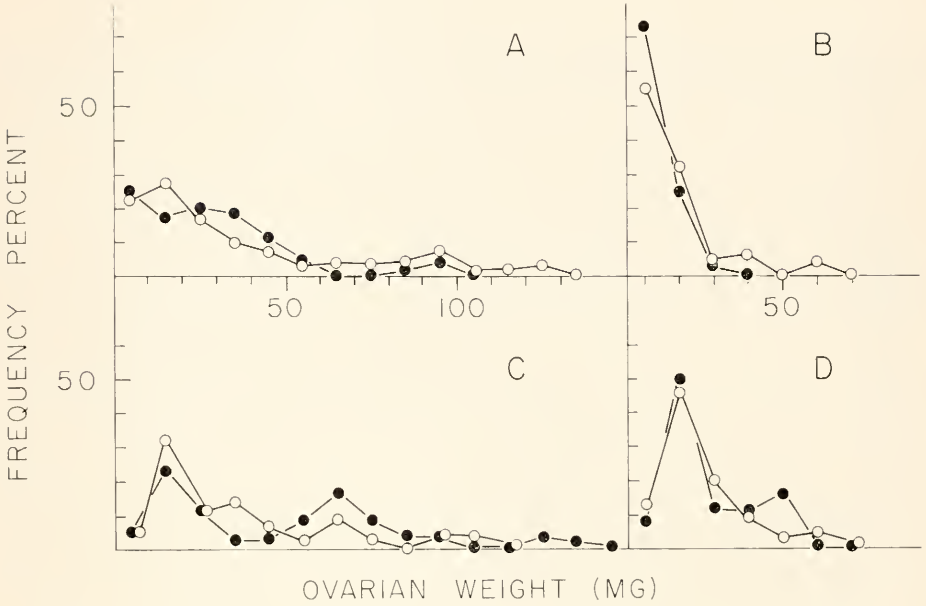

FIGURE 2. Frequency distributions by weight classes for crab ovaries: A, open circles, controls from February 25 shipment; solid circles, nonovigerous controls from April 15 shipment; B, open circles controls from January 13 shipment; solid circles, ovigerous controls from April 15 shipment; C, destalked from February 25 shipment; open circles 10L:14D; solid circles LL; and D, open circles, destalked from January 13 shipment; solid circles, destalked ovigerous from April 15 shipment.

with increasing weights, and no ovaries weighing above 60 mg. This represents a fairly normal J-shaped distribution. The crabs from the January shipment had ovaries in higher weight classes than those attained by ovigers in the April shipment. The distributions for crabs observed throughout the autumn are shown in Table II and are essentially like those illustrated in Figure 2B. The clustering in the smallest weight class was slightly more exaggerated in September, October and November, while in December 100% of the ovaries weighed less than 10 mg.

Three color classes are readily distinguishable on visual examination of the ovaries: purple, the color of mature ova, orange, infrequently present in control crabs, and colorless, characteristic of immature ovaries. The relative frequencies of occurrence of the three color classes for September through May are seen in Table III. It is apparent that the proportion of colorless ovaries in intact crabs is maximal from September through December. The proportion drops in January when the proportion of purple begins to increase. There follow precipitous drops in the proportion of colorless ovaries through March for all crabs and into April for nonovigers. For those that oviposited in mid-April, the proportion of colorless ovaries abruptly increases. For intact crabs the orange color occurs with maximum frequency (22%) in March and April.

The relative frequencies of molting in intact crabs are shown in Figure 1B. During September and October there were practically no molts, the frequency rose

TABLE II

Distributions for crabs observed throughout the autumn.

Observation dates	9/15 to 10/6		10/28 to 11/27		11/27 to 12/14		12/31 to 1/28	
Weight class (mg)	A	B	A	B	A	B	A	B
130–139.9	0	0	0	0	0	0	4	0
120	0	0	0	0	0	0	4	0
110	0	0	0	0	0	0	0	0
100	0	2	0	0	0	0	4	0
90	0	0	0	0	0	0	4	0
80	0	0	0	0	0	0	0	0
70	0	0	0	0	0	0	4	0
60	0	0	0	0	0	0	4	0
50	0	0	0	0	0	0	7	2
40	0	2	0	0	0	0	14	0
30	2	0	11	0	0	0	7	2
20	15	0	18	0	0	0	27	2
10	54	0	37	7	7	0	21	19
0–9.9	29	95	33	92	93	100	4	73

A = destalked crabs; B = intact crabs.

abruptly in November and still further in December, and dropped again during January through April. The ovigers which showed no molts from egg-laying until mid-May, showed a very high frequency of molts during the last half of May. Comparison of Figure 1B with Figure 1A makes it clear that there is a generally negative correlation between ovarian weights and molting frequency. Maximal molting coincides with minimal ovarian weights (December and late May), and there is a very low frequency of molt in March and April when ovarian weights are maximal. However, molting frequency is low in September and October when ovarian weights are also low and again in January when the ovaries are just beginning to show an increase in weight.

TABLE III

Relative frequencies of three color classes of ovaries.

	Controls				Destalked			
	C	O	P	Number of crabs	C	O	P	Number of crabs
September-October	96	0	4	75	4	85	11	67
November-December	95	0	4	56	7	84	9	43
January	74	7	19	42	0	34	66	41
February-March	39	10	51	100	4	54	41	44
March–April	19	22	58	136	12	48	39	66
Ovigers:								
April	75	11	14	45	0	61	39	38
May	86	0	14	14	25	56	19	24

C = colorless; O = orange; P = purple.

Effects of eyestalk removal

The effect of destalking on mean ovarian weights, expressed as differences from the controls, is shown in Figure 3A. Clearly the maximum effect is obtained in January, thus coinciding with very early ovarian maturation. The ovarian weights of destalked crabs are significantly different from those of the controls for September through January and again in April and May for ovigers, as judged by Student's *t*-test ($P < 0.05$).

In Table I are seen the gonadal indices, variances, and CW for destalked crabs. The CW ranges from 14.6 mm (September and December) to 16.0 mm (January). The gonadal index is higher than that for intact animals in all months and reaches its lowest value in December. The differences between gonadal index of intact and destalked crabs show the same general pattern as that shown by differences in ovarian weights.

Relative frequency distributions by weight classes for destalked crabs are shown in Figure 2C and D. The distributions in Figure 2C are for crabs received February 25. Those in Figure 2D are for crabs received January 13 and for the ovigerous crabs received April 15. Comparison with the control crabs for the same periods (Fig. 2A, B) reveals that following destalking there is a sharp reduction in the proportion of ovaries occupying the smallest weight class. The distributions for September through January are seen in Table II. The initiation of growth in originally immature ovaries is a consistent effect of destalking with variations related only to the proportions of completely immature ovaries found in the controls. The greatest change in distribution is seen in January (Table II) when destalked crabs show a distribution approaching in heterogeneity that seen for intact crabs in February and March.

One of the most conspicuous differences between ovaries of destalked and those of intact crabs is the increase in frequency of orange color in the former. The highest frequency occurs in the post-breeding months when no orange ovaries are found in the controls (Table III). The lowest frequency of orange in destalked crabs is found in January when the greatest weight increase occurs. At this time also the destalked crabs achieved the highest proportions of the purple color that characterizes the mature ovaries of intact crabs. In February and March, while mean ovarian weights still exceeded those of intact crabs, the proportion of purple ovaries actually decreased relative to the controls.

The frequencies of molting in destalked crabs relative to the controls are presented in Figure 3B. Maximum effectiveness in inducing molt occurs in September at a time when molt frequency is low in the controls (Fig. 1B), and again in April and early May for ovigerous crabs when no controls molted. In November, when 34% of the controls molted, there were slightly fewer destalked crabs that molted (27%). In late May both destalked post-ovigerous crabs and their controls molted at a frequency of about 42%.

Although there was no great difference between controls and destalked crabs with respect to survival, there were two times during this study when mortality was high for both groups. One time was in December and the other was in late May. Both are times when controls were undergoing ecdysis during the experimental period. In these cases the deaths occurred in ecdysis or just prior to ecdysis

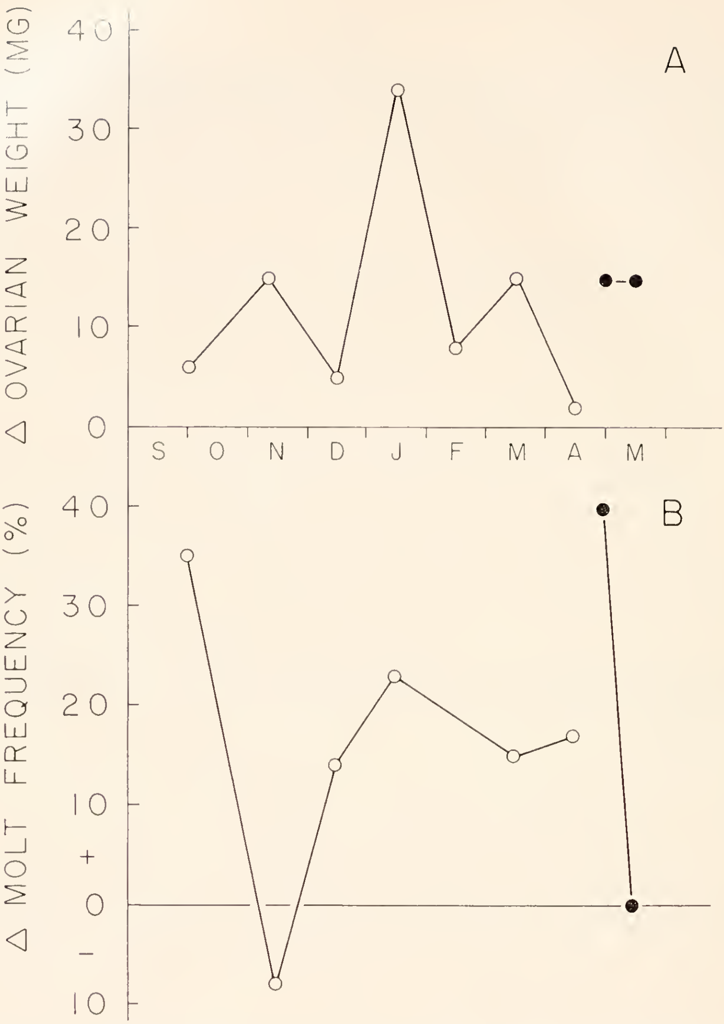

FIGURE 3. A, change in ovarian weights following eyestalk removal, expressed as difference from controls, as a function of time of year; and B, change in molt frequency following eyestalk removal, expressed as difference from controls, as a function of time of year. Open circles represent nonovigers; solid circle, April ovigers.

as revealed by autopsy. During the other times of year ecdysis was generally successful both for destalked and intact crabs.

Egg-laying was infrequent in the laboratory except during the period immediately following April 15. The earliest laying by intact crabs occurred in late March when two crabs laid eggs. For destalked crabs, one crab laid eggs 16 days after destalking in January, and one did so 18 days after destalking in February. Four destalked crabs oviposited in March at intervals of from 20 to 23 days after destalking. It was during this same four-day period that the intact crabs oviposited. No

Table IV

Occurrence of molt in experimental periods.

Observation period	Number of molts		First and last day		Median day	
	Experimental	Control	Experimental	Control	Experimental	Control
9/16–10/6	22	1	13–22	19	18	—
10/28–11/27	10	12	9–21	10–17	14	12
11/27–12/14	1	1				
	17*	9*	8–16	8–17	12	10
12/31–1/28	11	2	13–27	16–18	21	16
	5*	0	4–9	—	6	—
2/26–3/29	13	1	16–29	20	22	—
4/15–5/5	12	0	10–20	—	15	—

* Dead in ecdysis or very close to it.

nonovigerous crabs were destalked after April 15. All of the crabs that laid eggs in the laboratory attached the eggs to the pleopods in an apparently normal manner.

Discussion

Throughout the course of this study obvious changes in the condition of the ovaries, in molting frequency, and in the response of crabs to removal of eyestalks were observed. As with any laboratory study, a number of questions can be raised concerning the relevance of the observations to events in the field and the completeness of the sample studied.

For intact crabs, the overall pattern of ovarian conditions is one of decreasing weights from September through December, followed by a gradual increase from January through March, with first egg-laying occurring in mid-April. To what extent can these changes be explained on the basis of systematic differences in the size classes examined? While it is true that the minimal ovarian weight occurs at the time of minimal CW (December), the times of maximal ovarian weights do not correspond with those of maximal CW. A similar lack of correlation between gonadal indices and CW is seen to exist in Table I. It therefore seems that the pattern observed is not attributable to differences in size classes of the samples examined.

To what extent are the observed animals representative of those in the field? The crabs received in September, October and April were freshly collected, while those in December, January and February had been maintained in holding tanks for varying periods. It was seen in Figure 1A that the mean ovarian weights observed for freshly collected crabs that were not carrying eggs agreed well with the weights obtained for crabs received February 26 and observed in March and early April. Since these latter observations were made on crabs that had over-wintered in holding tanks, it seems that the holding conditions permit ovarian development essentially the same as that occurring in the field. The question of whether the samples examined were *completely* representative of the natural population is more involved. The observation of first egg-laying in mid-April agrees with the report of Salmon and Astaides (1968) for this species in the North Carolina area. It is consistent with the report of Herrnkind (1968) with respect to the beginning of active reproduction in Florida. However, the finding in this study that April ovigers molted before any further ovarian development occurred, as well as the absence of ovigers and of crabs with mature ovaries in September and October are inconsistent with Herrnkind's report of practically all females being in berry from May to October. Such a situation would imply that the crabs mature eggs while carrying one clutch and oviposit immediately after hatching. While it is possible that in autumn ovigerous females were absent from the collection but that some were present in the field, it is obvious that there were large numbers of nonovigerous females available for collection. Most of the crabs examined in this study for which the date of oviposition was known and which were examined within a week or ten days were found to have a few large purple or orange ova visible in the ovary. Only a very few of the crabs examined from the September or October shipments showed any evidence of recent oviposition. Since no observations were made between May 30 and September 15, it is not possible to say whether a molt follows each hatching that occurs throughout the summer. However, if the crabs received in September had been ovigerous as late as the end of August, it seems likely that the molt following the last oviposition of the season is delayed until November or December.

On the basis of the observations presented here, it appears that the active breeding season for crabs in Panacea begins in April and has concluded by mid-September. The crabs that oviposit in April represent about 30% of the population, and they molt in late May. Another third would be ready to oviposit by early May and the final third by late May or early June. The presence of ovigers at all times from April through August could be accounted for by a repetition of the sequence of oviposition-hatching-molt with the first ovigers of the season having completed their second vitellogenesis by mid or late June. Given such a pattern, a compression of the breeding season could be achieved by increased synchronization of ovarian maturation together with reduction in the number of times the sequence is repeated. A factor contributing to synchronization of the first oviposition at the northern limits of the range for *U. pugilator* might be the restraint placed on early vitellogenesis by low temperature.

The significance of the orange color in the ovaries is not clear. The normal purple color of mature ovaries of this species is that of a caroteno-lipoprotein which has been partially characterized by Wallace, Walker and Hauschka (1967). Absorption in the range of visible light is a function of solvent conditions, and the original purple color is readily changed to orange by a variety of factors, including pH. The alteration of color need not involve major structural change in the lipoprotein. Thus, the appearance of the orange color need not imply change in the synthetic pathway but may simply reflect general metabolic changes induced by eyestalk removal. Since the maximum development of the orange color in destalked crabs occurs when mean ovarian weights are decreasing in the controls, and minimally when they begin to increase, it is possible that the differences are related to varying amounts of ovary-stimulating hormone. For the destalked animals the relative frequencies of orange and purple in January approximate those seen for intact crabs in March and early April. January was also the time when the weight distributions for ovaries of destalked crabs were most different from those of their controls, and came to resemble the March distributions for intact crabs. Thus, in

January many crabs appeared to undergo a more complete vitellogenesis as a result of destalking than had occurred in the preceding months.

The effectiveness of destalking in inducing molt can be considered in terms of both increased frequency of molt relative to controls and the time required for molt to occur after the operation. Passano (1953) concluded that if molt occurred within ten days after eyestalk removal, the crab was in premolt when the operation was performed and molt could not be attributed to removal of eyestalk MIH. In this study the only experiment in which most of the molts occurred within ten days was that begun May 15, in which post-ovigerous crabs were destalked and 42% of both controls and destalked molted within ten days. In the other two periods when enough controls molted to make a comparison useful (November and December) both controls and experimentals were undergoing ecdysis at approximately the same time (Table IV). In neither of these two months does there seem to have been any effect of destalking so far as molt is concerned. On the other hand, for those months in which destalking did induce molt, there are variations in the time required for molt to occur. The median days of molt for January and March were days 21 and 22 after eyestalk removal, while for late April the median day is day 15. Very few controls molted in any of these months. This suggests that, although MIH is present in intact crabs in all three periods, the crabs destalked in April were closer to molt than were those destalked in January or March.

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SUMMARY

1. The condition of the ovaries of intact and eyestalkless *Uca pugilator* from Florida was examined throughout the period from mid-September to the end of May. The frequency of molt for the animals was also observed.

2. Ovarian weights for intact crabs decreased from September to December and began to increase in January. The increase did not occur uniformly throughout the population, and by March all stages of maturation were present. First oviposition occurred in April, involving about 30% of the females. No oviposition occurred as late as mid-September.

3. Crabs that oviposited in April did not exhibit further vitellogenesis prior to molt which occurred in late May. A high frequency of molting again occurred in intact crabs in November and December.

4. Removal of eyestalks led to initiation of vitellogenesis in immature ovaries at all times tested and also appeared to increase the rate of vitellogenesis in already maturing ovaries.

5. Removal of eyestalks increased the frequency of molt maximally in September and late April and caused no increase in November and December or for post-ovigers in late May.

6. The pattern of reproduction and molt for this species in the months studied appears to consist of the following sequence: vitellogenesis-oviposition-incubation-hatching-molt. The breeding season could be extended by repetitions of the sequence or compressed by increasing the degree of synchronization.

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